Novel Targets to Prevent Glucose-Induced Apoptosis and Dysfunction of Pancreatic β-Cells for the Treatment of Diabetes

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1. SUMMARY

Type 2 diabetes mellitus (T2DM) is characterized by chronic hyperglycemia that results from inadequate insulin secretion in the face of insulin resistance. The efficiency of β-cells to secrete sufficient amounts of insulin to maintain normoglycemia depends on β-cell function and β-cell mass. The endocrine pancreas has an exceptional capacity to adapt insulin output to an increased demand in conditions such as insulin resistance during obesity. However, in a subset of individuals at some point, this compensation fails and β-cells do not secrete enough insulin anymore to cope with the metabolic demand, leading to the development of type 2 diabetes. This disease is therefore a result of relative insulin deficiency.

Glucose has been identified as key fuel and regulator of β-cell performance and survival. It is not only the major nutrient that induces insulin secretion, but it is implicated as stimulus for the expansion of β-cell volume as a mechanisms of compensation, since it has been shown that glucose induces β-cell proliferation *in vitro*. However, glucose can also negatively affect function and survival. Chronically elevated glucose levels decrease function and induce β-cell death in isolated islets. These findings have led to the establishment of the concept of glucotoxicity and have raised the interest in finding mechanisms and targets to protect from hyperglycemia-induced diabetes progression.

It has been shown that obesity and type 2 diabetes are associated with low-grade inflammation indicating that immunological components are involved in the pathogenesis of this disease. The adipose tissue seems to be a major source of these inflammatory mediators. In obese individuals, adipocytes as well as infiltrating macrophages release increased amounts of non-esterified fatty acids, glycerol, hormones and pro-inflammatory cytokines that have been shown to play a crucial role in the development of insulin resistance. In addition to the occurrence of systemic sub-clinical inflammation, local inflammation in the pancretic islet has been identified as a major factor in the progressing deterioration of β-cells in T2DM. The pro-inflammatory cytokine Interleukin-1β (IL-1β) has been characterized as main active contributor. High glucose induces production and secretion of IL-1β by β-cells themselves, which in turn partly mediates the adverse effects of glucose. Therefore, IL-1β constitutes an interesting target in the search for a therapy for type 2 diabetes. A promising inhibitor of IL-1β is its natural antagonist Interleukin-1 Receptor Antagonist (IL-1Ra). IL-1Ra belongs to the IL-1 family and binds to IL-1 receptors but without inducing signal transduction, thereby antagonizing the inflammatory effects of IL-1β competitively. Indeed, blocking experiments *in vitro* have shown that IL-1Ra counteracts IL-1β-induced β-cell failure and death. Furthermore, a recent clinical trial demonstrated that IL-1Ra treatment improves glycemic control in type 2 diabetic patients. One part of this work aimed to further characterize the protective mechanisms of IL-1Ra on β-cells. Hence, we

investigated the ability of IL-1Ra to prevent diet-induced diabetes. Mice fed a high fat/ high sucrose diet (HFD) for 12 weeks developed impaired fasting glucose and hyperglycemia, which was prevented in animals from the HFD-group that were treated with IL-1Ra. Improvement was also observed during intraperitoneal glucose tolerance tests as IL-1Ra protected from impaired glucose clearance, which was a result of improved insulin secretion as well as insulin action. On the level of the β-cell, we found that IL-1Ra protected from apoptosis and enhanced glucose-stimulated insulin secretion of isolated islets. In parallel, high fat feeding induced a decrease in insulin mRNA, which was prevented by IL-1Ra administration. As an underlying mechanism of decreased function, we identified the insulin transcription factor PDX-1 (pancreatic duodenum homeobox-1) whose localization shifted from the nucleus to the cytoplasm after 12 weeks of HFD. IL-1Ra-mediated protection from β-cell failure was associated with PDX-1 remaining in the nucleus. We confirmed that the balance between glucose/ IL-1β and IL-1Ra also regulates PDX-1 localization in isolated human islets. Finally, we identified the JNK-pathway as possible link in the signal transduction between IL-1β/ IL-1Ra and PDX-1.

The second project of this work investigated the mechanisms of the loss of glucose-stimulated insulin secretion induced by long-term hyperglycemia, the so-called glucose desensitization. We hypothesized that this process is controlled by the group of extracellular signal regulated kinases (ERK)-1/2. ERK1/2 are universal kinases that, in β-cells, have been implicated to mediate short- as well as long-term effects of glucose. In the acute state, ERK1/2 induces insulin transcription and participates in the process of insulin secretion. In the long-term, ERK1/2-activity is involved in the adverse effects of chronic glucose incubations. We evaluated whether glucose desensitization after chronic hyperglycemia affects the responsiveness of ERK1/2 to a glucose challenge. We found that glucose-induced ERK1/2 phosphorylation is diminished; correlating with abolished stimulated insulin secretion. Interestingly, both inhibition of insulin release and ERK1/2-activity can induce a state of ‘β-cells rest’ despite prolonged exposure to elevated glucose concentrations. Inhibiting overstimulation sensitizes the islets for a following glucose stimulation resulting in increased insulin secretion as well as pERK1/2 upregulation. Hence, we confirmed a role of pERK1/2 in the deleterious effects of glucose on β-cells but, at the same time, demonstrate a correlation between the ability for an acute stimulation of insulin secretion to an increase in pERK1/2 levels.

In summary, in these studies both IL-1Ra- and ERK1/2-actions are analyzed related to glucose-mediated effects in β-cells and are recognized as key-players determining the fate of β-cells. Taken together, the results of this work identify possible novel therapeutic approaches by further characterizing the mechanisms underlying hyperglycemia-induced loss of β-cell function and mass.

2. ZUSAMMENFASSUNG

Der Typ 2 Diabetes Mellitus (T2DM) zeichnet sich durch chronische Hyperglykämie aus, die als Folge von mangelhafter Insulinsekretion in Kombination mit Insulinresistenz im Gewebe entsteht. Die Leistungsfähigkeit der β-Zellen, genug Insulin abzugeben, um einen physiologischen Blutzuckerspiegel aufrecht zu erhalten, hängt sowohl von der Insulinproduktion und dem Sekretionspotential als auch von der Masse der β-Zellen im Körper ab. Der endokrine Teil der Bauchspeicheldrüse hat eine enorme Kapazität, die Insulinabgabe an Bedingungen anzupassen, die einen erhöhten Bedarf hervorrufen, wie zum Beispiel an die erhöhte Insulinresistenz, die bei Fettleibigkeit entsteht. In einigen Fällen jedoch kann diese Kompensation versagen, sodass die β-Zellen nicht mehr genug Insulin freisetzen um der Anforderung des Metabolismus zu entsprechen, was dann zur Entwicklung von Typ 2 Diabetes führt. Diese Krankheit ist demzufolge das Resultat eines Missverhältnisses zwischen Insulinangebot und Insulinbedarf.

Glucose ist einer der Hauptfaktoren, der sowohl die Funktion der β-Zellen als auch deren Überleben beeinflusst. Glucose ist nicht nur der wichtigste Nahrungsstoff, der zur gesteigerten Insulinabgabe führt, sondern ist auch an β-Zellkompensationsmechanismen beteiligt. *In vitro* wurde gezeigt, dass Glucose β-Zellproliferation auslösen kann. Im Gegensatz zu diesen Eigenschaften kann Glucose die β-Zellen auch zerstören. Dauerhaft erhöhte Glucosekonzentrationen führen zu Minderleistung und Zelltod in isolierten Inseln. Diese Befunde werden unter dem Begriff ‚Glucotoxizität' beschrieben und haben ein grosses Interesse daran geweckt, Mechanismen und Komponenten dieses Prozesses zu finden, um ein weiteres Fortschreiten des Diabetes aufgrund vorhandener Hyperglykämie zu verhindern. Es wurde gezeigt, dass sowohl Fettleibigkeit als auch T2DM mit Entzündungsmechanismen verbunden sind, was darauf hindeutet, dass die immunologische Komponente einen Teil der Pathogenese dieser Krankheit darstellt. Dabei scheint das Fettgewebe eine wichtige Quelle entzündlicher Faktoren zu sein. Die Adipozyten übergewichtiger Menschen sowie auch ins Fettgewebe infiltrierende Makrophagen geben verschiedene Stoffe in erhöhter Konzentration ab. Dazu gehören nicht-veresterte Fettsäuren, Glycerol, Hormone und pro-inflammatorische Zytokine, von welchen gezeigt wurde, dass sie zur Entwicklung der Insulinresistenz beitragen. Zusätzlich zur vorhandenen systemischen, sub-klinischen Entzündung spielen lokale Entzündungsherde in den Inseln der Bauchspeicheldrüse eine entscheidende Rolle im fortschreitenden Versagen und Zelltod der β-Zellen in Patienten mit Typ 2 Diabetes. Das pro-inflammatorische Zytokin Interleukin-1β (IL-1β) ist dabei ein massgeblich aktiver Faktor. Erhöhte Glucosekonzentration regt die β-Zellen an, selbst IL-1β zu produzieren und abzugeben, was dann wiederum zur Selbstzerstörung der β-Zellen führt. Die entscheidende Rolle von IL-1β in der Pathogenese des Typ 2 Diabetes hat dieses Zytokin zu einem

interessanten Ziel in der Suche nach einer neuen Diabetestherapie gemacht. Ein vielversprechender, natürlich vorkommender Faktor, der die Aktivität des IL-1β blockiert, ist der Interleukin-1 Rezeptor Antagonist (IL-1Ra). IL-1Ra gehört zur gleichen Familie wie IL-1β und bindet an den gleichen Rezeptor (Interleukin-1 Rezeptor, IL-1R), löst aber keine Signalkaskade aus. Daher ist IL-1Ra ein Konkurrent von IL-1β, der Entzündungsreaktionen verhindert. Tatsächlich wurde in *in vitro* Experimenten gezeigt, dass die IL-1Ra-Zugabe zu isolierten Inseln IL-1β ausgelöstes β-Zellversagen und Apoptose abblocken kann. Desweiteren hat eine klinische Studie nachgewiesen, dass IL-1Ra-Behandlung die Insulinproduktion in Typ 2 Diabetikern verbessert. Eines der Projekte dieser Arbeit hat sich damit befasst, die Mechanismen von IL-1Ra, die zum Schutz der β-Zellen führen, weiter zu erforschen. Deshalb wurde die schützende Wirkung von IL-1Ra in einem Mausmodell genauer untersucht. In Mäusen löste eine 12-wöchige Nahrung mit hohem Anteil an Fett und Sucrose erhöhte Nüchternblutzuckerwerte sowie Hyperglykämien aus, eine Entwicklung, die durch tägliches Spritzen von IL-1Ra verhindert wurde. Verbesserungen wurde auch bei Glucosetoleranztests festgestellt, da IL-1Ra vor verzögertem Glucoseabbau im Blut schützte. Weitere Experimente zeigten, dass dies aus einer Kombination von verbesserter Insulinabgabe und Insulinwirkung hervorgerufen wurde. Auf der Ebene der β-Zellen wurde festgestellt, dass IL-1Ra sowohl Zelltod durch Apoptose als auch verminderte glucose-stimulierte Insulinsekretion verhinderte. Fett- und Sucrosereiche Nahrung führte zu erniedrigten Insulin mRNA-Konzentrationen, das ebenfalls durch IL-1Ra Behandlung vermieden wurde. Auf der Suche nach Mechanismen, die die β-Zellfunktion regeln, wurde der Insulin Transkriptionsfaktor PDX-1 (Pancreatic Duodenum Homeobox-1) gefunden, dessen Lokalisation nach 12 Wochen Fett- und Sucrosereicher Nahrung vom Zellkern ins Zytoplasma wechselte. Der durch IL-1Ra vermittelte Schutz vor β-Zellversagen war dabei mit dem Erhalt von nukleärem PDX-1 verbunden. Im letzen Teil dieses Projektes werden Hinweise geliefert, dass die Balance zwischen Glucose/ IL-1β und IL-1Ra auch in humanen Inseln die Lokalisation von PDX-1 bestimmt. Dabei wurde der c-Jun N-terminale Kinase (JNK)-Signalweg als Vermittler zwischen IL-1β/ IL-1Ra und PDX-1 gefunden.

Das zweite Projekt dieser Arbeit befasst sich mit den Konsequenzen des durch Hyperglykämien–ausgelösten Verlusts der glucosevermittelten Insulinsekretion, die sogenannte Glucose-Desensibilisierung. Unsere Arbeitshypothese war, dass durch extrazelluläre Signale regulierte Kinasen (ERK1/2) mit anhaltender Aktivierung einen Teil der negativen Einflüsse durch Glucose vermitteln. ERK1/2 sind universelle Kinasen, die in den β-Zellen sowohl kurz- als auch längerfristige Glucosesignale weiterleiten. Akut löst ERK1/2 die Transkription des Insulingens aus und nimmt auch am Prozess der Insulinsekretion teil. Wir untersuchten, ob Glucose-Desensibilisierung durch chronische Hyperglykämien die akute ERK1/2-Aktivierung beeinflusst. Unsere Resultate zeigen, dass β-Zell-Desensibilisierung zu

einer abgeschwächten ERK1/2-Aktivierung führt. Dies geht einher mit einer verminderten Stimulation der Insulinsekretion. Interessanterweise kann sowohl die Blockade der Insulinabgabe als auch die Hemmung der ERK1/2-Aktivität eine Art Erholungsphase in β-Zellen auslösen, auch wenn eine erhöhte Glucose-Konzentration über längere Zeit vorhanden ist. Diese Verhinderung der Überstimmulierung führt dazu, dass die Inseln sensibel genug sind für eine folgende Glucose-Stimulation um sowohl Insulin zu sezernieren als auch ERK1/2 Proteine zu phosphorylieren. Somit haben wir bestätigt, dass ERK1/2 eine wichtige Rolle in den schädlichen Wirkungen der Glucose auf β-Zellen spielen, zeigen aber gleichzeitig, dass deren Aktivierung als Antwort auf eine akut erhöhte Glucosekonzentration mit der Fähigkeit der Zellen, Insulin freizusetzen, verbunden ist.

Zusammengefasst analysieren diese Studien sowohl IL-1Ra als auch ERK1/2 in Bezug auf deren Rolle in β-Zellen als Vermittler von Glucoseeffekten. Dabei wurde festgestellt, dass beide Schlüsselfaktoren sind, die das Schicksal der β-Zellen mitbestimmen. Damit identifiziert diese Arbeit das Potential möglicher neuer Therapieansätze, indem sie weitere Mechanismen des Hyperglykämie-induzierten Versagens und Verlusts von β-Zellen aufklärt.

3. INTRODUCTION

3.1 The Pancreas

The pancreas is a gland organ about 6 inches (15 cm) long that stretches across the back of the abdomen consisting of exocrine and endocrine tissue (Fig. 1). The exocrine cells, called acinar cells, produce pancreatic juice containing digestive enzymes. These enzymes are secreted into a network of ducts and released into the intestine where they digest carbohydrates, proteins and fats. The endocrine cells form tight clusters called 'Islets of Langerhans' and are scattered throughout the pancreas forming approximately 1% of the total mass of the pancreas.

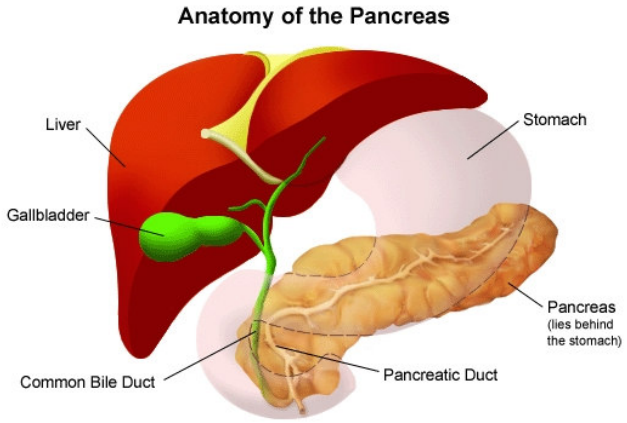

Figure 1: The pancreas. (Picture adapted from (2)).
The pancreas is located in the abdomen behind the stomach. The head of the pancreas is located on the right side of the abdomen, connected to the duodenum, the upper end of the small intestine. The narrow part of the pancreas, called the tail, extends to the left side of the body.

Although the endocrine tissue is a small portion of the whole pancreas, its hormones which are secreted directly into the bloodstream regulate many important aspects of a body's metabolism. The following cell types are present in the Islets of Langerhans:

β-cells secreting insulin
α-cells secreting glucagon
δ-cells secreting somatostatin
PP-cells containing pancreatic polypeptide

These hormones have several anabolic and katabolic effects in order to keep blood glucose concentrations in a narrow range. Insulin is released in response to several stimuli, the main one being elevated blood glucose levels. It acts on muscle, adipose tissue and liver by promoting glucose uptake and inhibiting glycogenolysis, thus returning high circulating glucose concentrations to normal values. If blood glucose levels are low, e.g. after exercise or in-between meals, α-cells produce glucagon who acts mainly on the liver. In response,

hepatic cells break down stored glycogen by a process called glycogenolysis. The resulting glucose is released into the bloodstream, preventing hypoglycemia. Somatostatin is an inhibitory peptide that regulates the endocrine system by suppressing the release of several gastrointestinal and pancreatic hormones. Pancreatic polypeptide is released after meal ingestion and suppresses food intake and gastric emptying as well as increases energy expenditure. Among many hormones which increase blood glucose levels, insulin is the only one which decreases blood glucose and regulates glucose homeostasis. Insulin is a peptide hormone that is synthesized as pro-insulin containing a B-, C- and A-chain (Fig. 2). Proteolytic cleavage removes the C-peptide and produces the mature insulin which consists of 51 amino acids of the B- and A-chain bound together by disulphide bonds. Insulin release from β-cells is regulated by systemic glucose levels, the islet invading nervous system as well as hormones secreted by different organs such as intestine and adipose tissue. Upon entering β-cells through the glucose transporter GLUT2, glucose undergoes glycolysis resulting in an increased ratio of ATP to ADP. This is followed by closure of ATP-controlled potassium (K^+) channels which leads to depolarization of the cell membrane and opening of voltage-controlled calcium (Ca^{2+}) channels. The raise in intracellular Ca^{2+}-concentrations causes exocytosis of insulin-containing secretory vesicles. Insulin acts on different organs, including muscle, adipose tissue and liver. It promotes glucose uptake, glycogen and lipid synthesis, and it inhibits glycogenolysis, gluconeogenesis, lipolysis and proteinolysis.

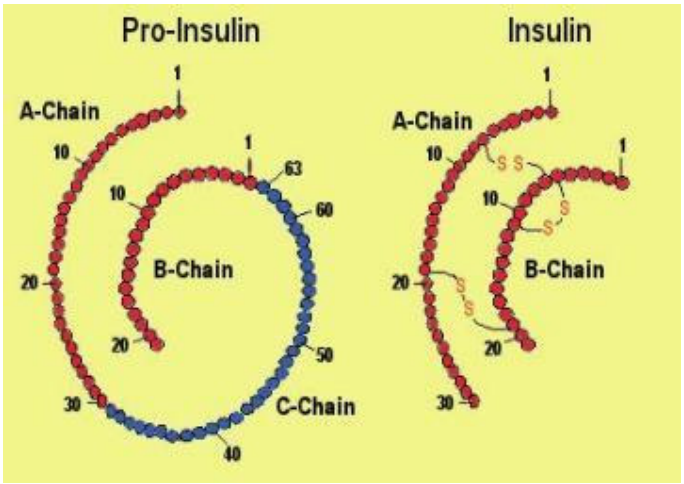

Figure 2: Pro-Insulin and Insulin. (Picture adapted from (1)).
Insulin is a polypeptide hormone, whose maturation includes cleavage at two positions to release the C-peptide as well as forming of three disulphide bonds. Its amino acid sequence is conserved, as the insulin from pigs differs in only 1 amino acid from the one produced in humans. It was the first protein whose structure was completely determined by Frederick Sanger, for which he was awarded the Nobel Prize in Chemistry in 1958.

3.2 Diabetes: Incidence and Etiology

Diabetes mellitus is a metabolic condition where the body fails to produce enough or properly use insulin to maintain blood glucose levels in the normal range. It is estimated that in 2006, 171 million people worldwide suffered from diabetes, a number reaching epidemic proportions and reflecting that this disease is likely to be one of the most substantial threats to human health.

Diabetes is a heterogeneous disease divided in several forms but all of them associated with high blood glucose concentrations (hyperglycemia). It is accompanied by macrovascular and microvascular complications which result in increased morbidity and mortality. As a consequence, diabetes may cause retinopathy leading to blindness, renal failure and neuropathy. It is also characterized by atherosclerotic damage which increases the risk for stroke, myocardial infarction and limb amputation due to poor wound healing.
The major types of diabetes are called type 1 (former childhood-onset or insulin-dependent diabetes) and type 2 (former adult-onset or non-insulin dependent diabetes).

3.3 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is an autoimmune disease resulting from specific destruction of pancreatic β-cells. The main cause of β-cell loss is considered to be autoimmune-mediated, with a minority of cases being idiopathic with no detectable immune markers. It has two phases: a moderate to long-term symptomless phase of insulitis when a mixed population of leucocytes invades the islets, and overt diabetes when most β-cells are killed off leading to absolute insulin deficiency and hyperglycemia. The incidence of type 1 diabetes is rapidly increasing in almost all populations and shows a trend towards earlier onset. Susceptibility for the disease is determined by environmental, genetic and immunological factors (6). Studies with monozygotic twin pairs revealed a concordance rate of T1DM of 30-50%, suggesting that at least 50% of the etiology are not due to genetic factors. Despite much research, it is not possible to pinpoint one particular environmental agent as causal factor and investigations focus on three groups of risk determinants: viral infections (e.g Coxsackie-B virus), early infant diet (e.g exposure to cow-milk) and toxins (e.g nitrosamines). Genetic studies have shown that this is a polygenic disease involving one major locus, the HLA class II region, as well as a number of non-HLA loci. The function of the genes of the HLA locus in terms of immune response is known, such as antigen presentation to T-cells, but the specific contribution to the pathogenesis is still under investigation. One of the non-HLA suscpetibility genes so far identified is the IDDM2-locus with a variable number

of tandem repeats 5' of the insulin gene and genome-wide association studies continue to identify other susceptibility variants (7). Even though B-cells, macrophages and dendritic cells have important pathogenetic functions, type 1 diabetes is considered to be a T-cell mediated disease. T-cells have been found to respond to several autoantigens including peptides derived from insulin and glutamic acid decarboxylase (GAD). The initial step in the development of T1DM is thought to be macrophages and/ or dendritic cells presenting β-cell autoantigens to CD4+ T-helper cells on MHC class II molecules. Activated macrophages and CD4+ T-cells release cytokines including interleukin (IL) -1β, tumor necrosis factor (TNF) -α and interferon (IFN) -γ. These attract and activate CD8+ T-cells in the inflammed islets which destroy β-cells by different mechanisms such as release of granzymes and perforin as well as by Fas - Fas-Ligand interactions (8). Even though massive specific β-cell destruction has been shown to be the hallmark of T1DM, underlying causes and mechanisms of β-cell death vary and affected individuals have heterogenous clinical parameters. Therefore, diabetes could be a set of related disorders with possible differences in initiating antigens, primary effector cell types and induction of β-cell death (9).

3.4 Type 2 Diabetes Mellitus

In type 2 diabetes mellitus (T2DM), pancreatic β-cells fail to produce enough insulin to meet the body's metabolic demand (10; 11). The disease is considered to result from the occurrence of increased insulin resistance together with decreased β-cell function and mass (4).

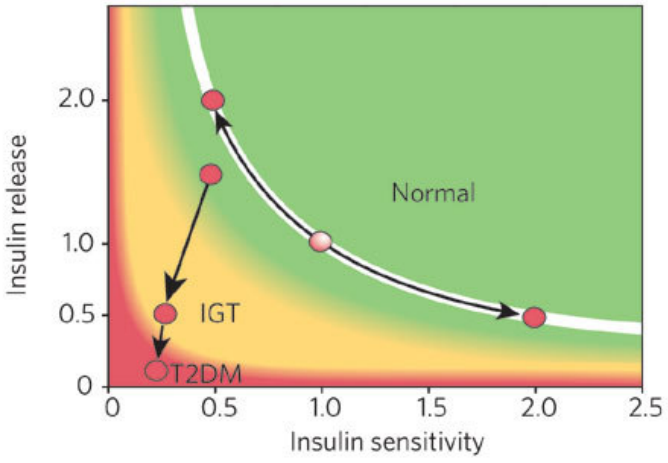

Figure 3: Relationship between insulin sensitivity and insulin release. (Picture adapted from Kahn et al. 2006, (4)).
The relationship between insulin sensitivity and insulin release is hyperbolic. Hypothetical regions delineating normal glucose tolerance (NGT, green), impaired glucose tolerance (IGT, yellow) and type 2 diabetes mellitus (T2DM, red) are shown. In response to changes in insulin sensitivity, insulin release increases or decreases reciprocally to maintain NGT, 'moving up or down' the curve. In individuals who are at high risk of developing type 2 diabetes, the progression from NGT to T2DM transitions through IGT and results in 'falling of the curve'.

Type 2 diabetes accounts for as much as 95% of diabetes cases (12) and modern lifestyle, with abundant nutrients and reduced physical activity, has led to a dramatic rise in the incidence of this obesity-associated condition. The overall risk and the rate of progression of type 2 diabetes is determined by genetic and environmental factors. T2DM is a polygenic condition, as various polymorphisms have been found within genes regulating islet function, insulin action and muscle, hepatic and adipocyte metabolism. Each variation adds risk and contributes to the genetic predisposition. In addition, susceptibility is also influenced by factors such as overnutrition (13), low birth weight (14), former gestational diabetes (15), a history of polycystic ovary syndrome (16) and being a first-degree relative of individuals with T2DM (17). Studies with some of these groups at high risk to develop type 2 diabetes have demonstrated that there is a whole spectrum of abnormalities, with some having reductions in insulin sensitivity, others having defects in β-cell function and most having abnormalities in both. Interestingly, these subjects show reduced β-cell function at a time when fasting plasma glucose concentration is still well within the normal range (18).

Progression to diabetes has been proposed to have five stages (19): Stage 1 is compensation where insulin secretion increases to maintain normoglycemia (~ 4.5 mM, fasting state) in the face of insulin resistance. The mechanisms are not fully understood, but both expansion of β-cell mass (20; 21) and enhanced β-cell function are implicated (22; 23). Stage 2 occurs when glucose levels rise to 5 – 6.5 mM, a stable state of β-cell adaptation that can last for years but is already accompanied by loss of β-cell mass and acute glucose-stimulated insulin secretion (24). Stage 3 is an unstable, transient stage where, due to early decompensation, glucose levels rise over a relatively short period of time to overt diabetes of stage 4. In most cases, this stage lasts a lifetime for patients with type 2 diabetes, as they typically have enough insulin secretion left rather than progressing to ketoacidosis. In contrast, rapid progressive autoimmune destruction of β-cells in type 1 diabetes can lead to stage 5, where the marked loss of β-cells is so severe that people become ketotic and truly dependent on insulin for survival.

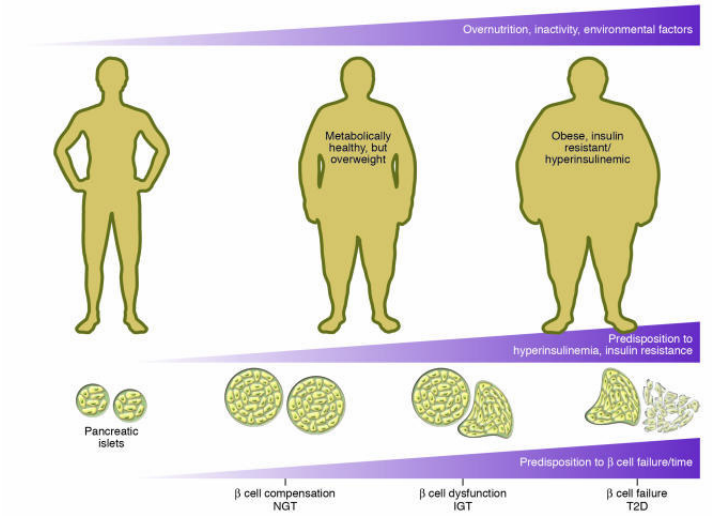

Figure 4:Development of type 2 diabetes. (Picture adapted from Prentki et al., 2006, (3)).
T2DM develops in response to overnutrition and lack of physical activity in individuals that have underlying genetic and acquired predispositions to both insulin resistance and β-cell dysfunction. Over time, islet β-cell compensation for the insulin resistance fails, resulting in a progressive decline in β-cell function. As a consequence, subjects progress from NGT to IGT and finally to established T2DM. β-cell function continues to worsen such that subjects progress from needing changes in diet and excersie to requiring oral hypoglycaemic agents or even insulin to achieve adequate glycaemic control.

As described above, two processes on the level of β-cells have been implicated in the development of diabetes: β-cell secretory failure and loss of β-cell mass.

β-cell secretory failure: Insulin secretion from patients with type 2 diabetes show a prominent loss of acute glucose-stimulated insulin secretion with a completely diminished first (24) and only partially preserved second phase (25). However, insulin response to nonglucose secretagogues, such as arginine, remains largely intact (26). T2DM is also associated with impaired ultradian and diurnal (meal-related) oscillatory patterns and with perturbed rapid pulsatile insulin secretion. All of these pulsatile defects are implicated in diminished insulin action and are considered to be an early marker for T2DM (27-31). In addition, a defective conversion of pro-insulin to insulin leads to elevated pro-insulin to insulin ratio where pro-insulin only has 10% of the activity of mature insulin. Together, the defective insulin secretion and insulin deficiency compromise the ability of β-cells to compensate for the increased demand due to insulin resistance which leads to hyperglycemia.

Decreased β-cell mass: β-cell mass is plastic and adjustements in β-cell growth and survival maintain a balance between insulin supply and demand. For example, during pregnancy, β-cell mass increases in adaptation to changes in metabolic homeostasis. In rats, β-cell population can almost double and proliferation coincides with an increase in placental lactogen and in prolactin and growth hormone receptors (32).

More importantly, β-cell mass adapts to an increased metabolic load caused by obesity and the inherent insulin resistance and therefore plays a crucial role in compensation. However, in a subset of individuals (~ 30%), this adaptation fails and type 2 diabetes develops. A study investigating human pancreata from autopsy revealed a 40% loss of β-cell mass in subjects with impaired fasting glucose (IFG) and a 63% loss in patients with T2DM compared to weight matched controls, respectively. Lean diabetic patients had loss of 41% of β-cell volume compared with lean controls. Furthermore, the decreased β-cell mass was found to be associated with increased β-cell apoptosis. The fact that loss of β-cell volume was evident in individuals with IFG suggests that this decrease is not only confined to late-stage T2DM (33). Many mechanisms could trigger the increase in β-cell apoptosis that occurs during the pathogenesis of T2DM. Among them are the development of endoplasmatic reticulum (ER) stress, oxidative stress, chronic hyperglycemia, dyslipidemia, and systemically and locally increased levels of cytokines.

3.5 Glucotoxicity and Cytokines as Mediators of β-Cell Secretory Failure and Apoptosis

Glucose is a key physiological regulator of β-cell function and survival whose outcome depends on concentration and duration of exposure. Studies on isolated islets from different species revealed that short-term exposure to elevated glucose concentrations induces insulin secretion as well as proliferation. However, prolonged increased glucose levels decreases function and moreover, lead to β-cell apoptosis (34-41). These findings imply a crucial role of hyperglyemica in the progression of diabetes. It suggests a detrimental effect of even only slightly elevated circulating glucose levels in individuals before the onset of overt diabetes. Furthermore, in type 2 diabetic patients on diet and/ or oral hypoglycemic agents, fasting glucose and HbA1c levels might be within the normal range, but post-prandial concentrations of glucose are often abnormal which could have continuing adverse effects on β-cells.

Chronic exposure to hyperglycemia and its consequences on β-cell function and survival have been described with three different terms (42-46): Glucose desensitization is defined as a temporary physiological state of cellular refractoriness to glucose stimulation induced by repeated or prolonged exposure to high glucose concentrations, which is reversed upon restoration of euglycemia. This process involves an intrinsic and reversible alteration in stimulus-secretion coupling. β-cell exhaustion describes a physical depletion of insulin stores following prolonged, chronic stimulation with glucose or other secretagogues. Insulin secretion is not possible, even if the β-cell would become resensitized to glucose. Glucose toxicity of the islets refers to nonphysiological and potentially irreversible cellular

damage caused by chronic exposure to supraphysiological glucose concentrations. It is characterized by defective insulin gene expression (47). So far, several mechanisms and pathways of β-cell biology are being investigated in terms of finding triggers of hyperglycemia-induced β-cell apoptosis and dysfunction.

One such trigger might be increased endoplasmatic reticulum (ER)-stress (48). In β-cells, the ER is highly developed due to heavy engagement in insulin secretion. Insulin resistance in peripheral tissue induced by obesity and physical inactivity leads to a chronic challenge to the capacity of β-cells and alterations in ER-function resulting from overload of proteins or accumulation of folding-incompetent proteins have been implicated in β-cell death and dysfunction (49; 50). An intrinsic program called unfolded protein response (UPR) adapts the ER to this stress situation, but finally induces apoptosis if this mechanism fails to clear the pertubation and ER-stress persists. (51). A mutation in the insulin gene leading to misfolded proteins has been shown to lead to β-cell death and diabetes in the Akita mouse (52) supporting a causative connection between ER-stress and diabetes. In addition, mediators of ER-stress have been found to be increased in pancreatic sections of type 2 diabetic subjects (53) and exposure to increased glucose levels induced ER-stress markers in islets from type 2 diabetes patients but not in non-diabetic controls (54).

Overstimulation by hyperglycemia might also result in chronic increases in cytoplasmatic [Ca^{2+}]. Chronically increased [Ca^{2+}] stands in contrast to the normal short-term rise in response to glucose metabolism that leads to fusion of insulin-containing vesicles to the cell membrane. These abnormalities in Ca^{2+}-influx result in aberrations in insulin oscillation and persist even after normalization of glucose levels, triggering apoptosis and therefore participating in the deterioration of β-cells (55).

Signaling networks activated by death receptors are not only involved in β-cell destruction in T1DM but are also detectable in islets from T2DM patients. Particularly, Fas-FasL interactions have been shown to play a role. Fas (also known as CD95) is a receptor of the TNF-superfamily that contains an intracellular death domain (DD) responsible for transduction of apoptotic signals. The pathway is activated by FasLigand (FasL) which leads to recruitment and proteolytic cleavage of caspase-8 through the DD, triggering the downstream cascade (56). β-cells have been shown to express Fas in response to cytokines (57-60) as well as to chronically elevated glucose (40). In addition, its expression is upregulated in type 2 diabetes islets, all of which renders β-cells susceptible to apoptosis by interaction with FasL on T-cells or neighbouring β-cells. Despite its role as inducer of apoptosis, Fas has also been implicated in proliferative signals in T-cells (61) and β-cells (62). The determinant whether activation of Fas leads to apoptosis or proliferation seems to be FLICE-inhibitory protein (FLIP). FLIP structurally resembles caspase-8 but lacks its proteolytic activity (63). In human islets, FLIP is constitutively expressed but decreased in

pancreatic tissue from T2DM patients and as well as after prolonged exposure of isolated islets to high glucose levels. Overexpression of FLIP protects β-cells from glucose-induced apoptosis and counteracts a glucose-induced decrease in proliferation and acute insulin secretion (62). Therefore, the Fas-FLIP system seems to be a key regulator of β-cells and might in part explain the dual role of glucose on β-cell turnover and function.

As described above, diminished insulin expression as a result of irreversible cellular damage is a characteristic of glucotoxicity (47). It has been shown *in vitro* and *in vivo* that loss of insulin production is accompanied by decreased expression and DNA binding activity of pancreas duodenum homeobox-1 (PDX-1) (64-66). The fact that treatment with antioxidants such as N-acetylcysteine improved glycemic control by preserving expression of PDX-1 and insulin suggests that glucotoxicity is in part explained by chronic oxidative stress (67-69). Already in 1987, it was proposed that metabolising excess glucose present during hyperglycemia can generate reactive oxygen species (ROS) in β-cells such as hydrogen peroxide or superoxide (70; 71). Uncoupling of oxidative phosphorylation by activation of uncoupling protein-2 (UCP2) decreases ROS-production but also reduces ATP synthesis and therefore results in impaired insulin secretion. In addition, β-cells only have limited defense against excess ROS-production compared to other tissue due to relatively small amounts of antioxidant enzymes such as catalase and glutathione peroxidase (72-74), rendering them at high risk for ROS-induced damage. Tanaka et al. (75) showed that high glucose concentrations indeed increased intracellular peroxide levels in the β-cell line HIT-T15 and in isolated human islets and that this increase was dependent on glucose metabolism. Several *in vitro* and *in vivo* studies so far observed a protective effect of antioxidative drugs on β-cells in the presence of chronically elevated glucose levels (43; 76-79). In addition, various reports on elevated markers of oxidative stress in the plasma and urine of type 2 diabetic patients provide clinical evidence that these mechanisms may contribute to β-cell dysfunction and death (80-84).

Over the last decade, an hypothesis of an immune origin of type 2 diabetes has been postulated with inflammatory mediators as a common link between type 1 and type 2 diabetes. Environmental factors such as high-caloric diet and insufficient exercise have been proposed to be major contributors to the pathogenesis of T2DM and also have a direct impact on levels of systemic immune mediators. These mediators are considered to be responsible for the effects of these environmental factors on insulin resistance and β-cell failure, forming an essential step in gluco- and lipotoxicity. Sub-clinical, low-grade inflammation has been observed in patients with type 2 diabetes and abnormalities of several systemic indicators of inflammation have been reported. These are hypothesized to not only represent markers of metabolic aberrations in T2DM, but may also contribute to β-dysfunction and death. Increased levels of acute phase proteins (e.g C-Reactive Protein

(CRP)), systemic cytokines (e.g IL-6, TNF-α) and factors of blood and endothelial cell activation (e.g ICAM-1, PAI-1, leucocyte count) have been found in diabetic patients. However, the extent of the observed immune activations is far lower as compared to those seen in acute infections. Since low-grade inflammation precedes type 2 diabetes by many years and is already present in subjects with IGT at the same levels as in individuals with overt diabetes, it is considered to be a contributor rather than a consequence of the disease (85). In addition, prospective studies have reported modest increases many years before the diagnosis of T2DM and importantly, even at a stage when most individuals were not expected to have IGT or IFG yet (86-91). Furthermore, there is evidence that not single cytokines seem to be important but rather that the pattern of circulating inflammatory mediators modify the risk for future type 2 diabetes. Spranger et al (90) found a significant interaction between IL-1β and IL-6, as only the combination of detectable levels of IL-1β and elevated IL-6 resulted in an increased risk to develop T2DM, whereas undetectable IL-1β and elevated IL-6 did not. The findings that alterations in cytokine levels modulate the risk for T2DM are supported by genetic studies which continue to identify associations between variants of immune genes and the risk to develop the disease. These alleles are found in several loci such as the HLA loci and the TNF-α, IL-6, CRP and the plasminogen activator inhibitor-1 (PAI-1) genes (92-95) which encode key modulators for an immune response and partly determine the susceptibility for diabetes. Several animal models also provide a link between those inflammatory genes and type 2 diabetes, as disruption or overexpression had a major effect on the risk to develop diabetes in response to a high fat diet (96; 97).

One major question is whether, and if so, how are immune mediators involved in β-cell failure. Maedler et al. (98) provided evidence that interleukin-1β (IL-1β) might partly be responsible for β-cell damage. *In vitro* exposure of islets from nondiabetic organ donors to prolonged elevated glucose levels resulted in increased RNA and protein levels as well as secretion of IL-1β into the supernatant, and β-cells themselves were found to be the islet cellular source of glucose-induced IL-1β. Long-term glucose or IL-1β treatment of islets resulted in Fas upregulation and NF-κB activation, followed by impaired insulin secretion and DNA fragmentation. These detrimental effects could be partly inhibited by adding Interleukin-1 receptor antagonist (IL-1Ra, a naturally occurring antagonist of IL-1β) or PDTC (NF-κB-inhibitor) to the culture medium. The importance of IL-1β *in vivo* was confirmed by detection of this cytokine in pancreatic sections of diabetic *psammomys obesus* as well as sections of type 2 diabetic patients, but not in non-diabetic controls, respectively. These findings implicate an hyperglycemia-induced inflammatory process in the pathogenesis of type 2 diabetes and identify IL-1β as mediator of glucose-induced adverse effects.

IL-1 is a prototypic pro-inflammatory cytokine that affects almost every cell type and often acts in concert with other cytokines such as TNF-α. There are two forms of IL-1 with

often indistinguishable activities, IL-1α and IL-1β. The 3rd member of the family is IL-1Ra, which is structurally similar but lacks the agonistic activity. There are two surface binding proteins for all three cytokines, the IL-1 type I receptor (IL-1RI) and the IL-1 type II receptor (IL-1RII).

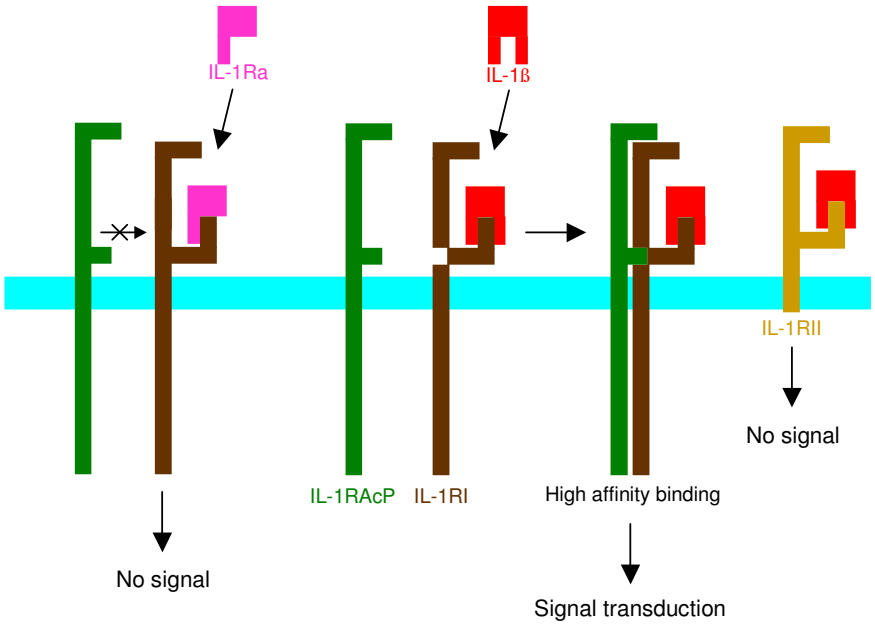

Figure 5: The IL-1 system. (Picture adapted from Dinarello et al., 1997, (5)).
Following binding of IL-1β to IL-1RI, there is a structural change of this receptor, allowing the formation of a complex with IL-1RAcP which results in signal transduction. Lacking the second binding domain, IL-1Ra binds to IL-1RI but does not result in a structural change of the receptor. Since it does not recruit IL-1RAcP, there is no signal transduction and no biological response. IL-1RII lacks a cytosolic segment, hence binding of IL-1β does not lead to signaling. Therefore, IL-1RII as well as the soluble forms of the receptors (not shown) act as 'decoy' receptors or a' sink'.

When IL-1 binds to the surface IL-1RI, the IL-1R accessory protein (IL-1RAcP) is able to dock and form a complex that triggers the intracellular downstream signal. Binding of IL-1 to IL-1RII does not transduce any signal since this receptor lacks the cytosolic domain, which makes it a "decoy" receptor. IL-1 and IL-1Ra may also bind to soluble extracellular domains of both receptors found in the circulation and in extracellular fluids after proteolytic cleavage, which function as natural "buffers". IL-1Ra has an amino acid homology of 26% to IL-1β and binds with almost the same affinity to IL-1RI. However, since it lacks a second binding domain and does not recruit IL-1RAcP, binding does not result in signal transduction. Therefore, IL-1Ra is unique in cytokine biology as it represents a naturally occurring antagonist to IL-1β. Hence, IL-1β activity is tightly regulated not only on the level of synthesis and secretion, but also through surface decoy receptors, soluble receptors and an antagonist (reviewed in (5; 99)).

The ability of IL-1Ra to inhibit IL-1β activity has been used in different clinical applications to reduce disease severity. Especially, chronic inflammatory syndromes with

increased levels of IL-1β respond to IL-1Ra treatment, for example systemic onset juvenile idiopathic arthritis ((100), commentary in (101)). IL-1Ra is induced by many of the stimuli that induce IL-1β in the same cell, such as immune cells, hepatocytes and adipocytes. As with IL-1β, IL-1Ra has been shown to be expressed by β-cells, and in contrast to IL-1β, its levels are decreased in pancreatic sections from type 2 diabetic patients (102). This is in line with a study showing that patients with type 2 diabetes have significantly lower plasma levels of IL-1Ra than non-diabetic patients (103). *In vitro*, addition of IL-1Ra to the culture medium or adenoviral overexpression (Ad-IL-1Ra) prevents glucose- and IL-1β-induced β-cell dysfunction and apoptosis (98; 104; 105), whereas depletion of IL-1Ra with small interfering RNAs (siIL-1Ra) results in impaired function, caspase-3 activation and DNA fragmentation. Infecting islets with Ad-IL-1Ra before transplantation into streptozotocin-(STZ)-rats increases β-cell replication and mass in the islet grafts and lowered apoptosis compared to uninfected islets. These beneficial effects led to an improved metabolic outcome, accelerated recovery of normoglycemia and reduced the amount of islets needed to consistently achieve normoglycemia (106). IL-1β has been postulated to contribute to the non-specific inflammation at the grafted site that damages transplanted islets even in the absence of rejection or autoimmune attack (107). Furthermore, IL-1β gene expression is increased over 4-fold in islets 24 hours after isolation and 45-fold in islets 1 day after transplantation (106). As a consequence, IL-1Ra might be an useful strategy to increase survival of transplanted islets and reduce the islet number required to reach normoglycemia.

The importance of a balance between IL-1β and IL-1Ra has been demonstrated *in vitro* and *in vivo* in several studies and is considered to be an important factor influencing the course, susceptibility to and severity of many diseases (108). In a healthy population, the ratio of plasma IL-1β to IL-1Ra is close to 1 with minimal variations (109). However, expression of these cytokines are influenced by gene polymorphisms and associations between allele variants and clinical outcome of various disorders have been found, including nephropathy in diabetes (110). The human IL-1Ra gene (IL1RN) has a variable number tandem repeat (VNTR) within intron 2 and so far, six alleles have been identified representing of 1 to 6 copies of a 86bp sequence (111), with the 4-repeat (IL1RN*1) and 2-repeat (IL1RN*2) variants being the most common ones, constituting 95% of all cases. The number of the repeats may have functional significance because it contains possible transcription factor binding sites (112). In the IL-1β gene, there are at least two biallelic base exchange polymorphisms, one at position –511 (in the promoter region) and one at position +3953 (within exon 5). Certain combinations of these polymorphisms have been shown to be significantly associated with T2DM (113). Also, healthy carriers of IL1RN*2 had higher levels of serum IL-1Ra than healthy non-carriers (745 vs 627 pg/ml). However, it has been shown that the enhancing effect of allele 2 on IL-1Ra plasma levels is influenced by the IL-1β-

variant present (114). It has also been demonstrated *in vitro* that the balance of IL1-β to IL-1Ra is crucial for β-cell survival and function (115). Even though IL-1β has detrimental effects on islets at doses of 2-5 ng/ml, a lower concentration of 0.2 ng/ml induces proliferation. However, the presence of endogenous IL-1Ra is required for this effect since repressing it using siIL-1Ra was again deleterious to β-cells. Since IL-1Ra does not have a signaling function itself, it is believed that the effect on β-cells is a result of the overall ratio of IL-1β to IL-1Ra.

A recent clinical trial has investigated IL-1β as a therapeutic target whose inhibition may preserve β-cell mass and function (116). Type 2 diabetic patients received daily subcutaneous injections of 100 mg of a recombinant human IL-1Ra for 13 weeks. The IL-1Ra-group showed improved glycemia due to improved β-cell secretory function, which was apparent as enhanced C-peptide secretion and reduced ratio of pro-insulin to insulin, compared to the placebo-group. In addition, IL-1Ra treatment also decreased systemic IL-6 and CRP, both markers of systemic inflammation. This study emphasizes IL-1β as a possible target and identifies the potential of IL-1Ra in treatment of type 2 diabetes. Part of the present study aimed to further characterize the role of IL-1β and IL-1Ra in the development of T2DM and investigate its ability to prevent diet-induced diabetes as well as find underlying mechanisms.

3.6 Obesity and Diabetes

The increased prevalence of obesity has focused attention on a world-wide problem, especially as obesity among children shows an equally marked increase (117). Obesity is associated with an array of additional health problems, including increased risk of insulin resistance, type 2 diabetes, hypertension, stroke, fatty liver disease and atherosclerosis as well as neurodegeneration, airway disease and some cancers (118). Environmental factors are largely responsible for the modern day epidemic of obesity. Increased caloric availability and fat consumption in the setting of decreased physical activity lead to overnutrition, increased nutrient storage and obesity. Between 60% and 90% of cases of type 2 diabetes now appear to be related to obesity (119). In addition to energy storage and metabolic homeostasis, the adipose tissue is considered to be an endocrine and paracrine organ, as it modulates a body's metabolism be releasing non-esterified fatty acids (NEFAs), glycerol, hormones and pro-inflammatory cytokines (120). The secretion of many of these products is increased in obesity and type 2 diabetes, linking metabolic diseases to inflammatory processes. An abundance of evidence has emerged demonstrating a relationship between metabolism and the immune system. From an evolutionary point of view, survival depends

on the ability to fight pathogens as well as to withstand starvation. Metabolic and immune systems are therefore among the most basic requirements that are also highly conserved. It is not surprising that metabolic and immune pathways have evolved which are closely linked and interdependent and are regulated through common key mediators and signalling systems. In addition, it is noteworthy that the adipose tissue has an architectural organization in which the metabolic cells (adipocytes) are in close proximity to immune cells (macrophages) and therefore constitutes a key site for the interaction of adipocytes and the immune system. It has been proposed that the current lifestyle of metabolic overload disturbes the fine balance between those two systems leading to an association between metabolic diseases, such as obesity and type 2 diabetes, and chronic inflammation (118). The finding that the pro-inflammatory cytokine TNF-α is overexpressed in the adipose tissue of obese mice provided the first molecular link between obesity, diabetes and inflammation (121). Furthermore, TNF-α expression is also increased in adipose and muscle tissue of obese humans (122-124). Moreover, various other inflammatory mediators and cytokines are upregulated in experimental mouse models of obesity and in humans, such as IL-6, Leptin and Resistin (125). The observation that the systemic levels of many of these molecules strongly correlate with body mass index (BMI), fat mass and/ or waist circumference (126) has led to the assumption that they originate from adipocytes directly. However, recent studies have revealed that adipose tissue infiltrating macrophages are a major source of inflammatory mediators (127; 128) and account for most of TNF-α and IL-6 expression. Macrophage infiltration has been described in obese conditions in both mice and humans and is thought to occur in response to chemotactic signals secreted by expanding adipocytes (125). Since pro-inflammatory gene expression in macrophages occur before systemic insulin is increased (sign of existing insulin resistance), studies especially focus on the role of chronic inflammation in promoting insulin resistance associated with obesity. Insulin resistance is an impairment of insulin action through interference of the insulin signaling pathway. Insulin signaling is initiated by binding to insulin- and IGF-receptors. The receptor tyrosine kinases then phosphorylate intracellular substrates that dock to these receptors. Among a large number of substrates, six belong to the family of insulin receptor substrate (IRS) proteins. Tyrosine phosphorylation of IRS is a crucial step in insulin action that is often defective in cases of insulin resistance. Inflammatory mediators as well as chronic hyperglycemia can also lead to serin/threonine phosphorylation of IRS which results in subsequent proteosomal degradation (129; 130). These IRS-modifying enzymes not only modulate insulin receptor signaling, they are also partly implicated in promoting further inflammatory processes, for example by activating NF-κB (131). Among them, the c-Jun N-terminal kinase (JNK) has been proposed to be central in mediating insulin resistance. JNK can be induced upon exposure to cytokines such as TNF-α (125) and to free fatty acids. In

obesity, JNK activity is upregulated in adipose and liver tissue (132). Furthermore, the absence of JNK1 results in decreased adiposity, significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity, supporting the concept that the development of insulin resistance and T2DM involves immunological components. Taken together, obesity promotes both chronic low-grade inflammation and insulin resistance and evidence suggests that type 2 diabetes is an inflammatory disease with inflammation as a primary cause rather than a consequence (125).

Despite the strong evidence pointing to obesity as major cause of insulin resistance, the fact that BMI and insulin sensitivity in healthy subjects do not have a linear correlation points to the presence of other factors contributing to insulin resistance. Indeed, it has been found that insulin sensitivity can be influenced by genetics (133), age (134; 135), acute exercise (136), physical fitness (137), dietary nutrients (138), medications (139) and body fat distribution (140-145) with central accumulation being the critical determinant of reduced insulin sensitivity (18).

However, not all obese, insulin-resistant individuals develop diabetes. As described above, β-cells are markedly plastic in their ability to regulate insulin release to maintain normoglycemia, both through increased secretory capacity (can be 4- to 5-fold enhanced) and increased β-cell mass (can be increased up to 50%) (33; 146). In healthy individuals, this response of a proportionate and reciprocal alteration in insulin output results in glucose tolerance remaining normal (Fig. 3). Therefore, the two parameters of β-cell function and insulin sensitivity must always be assessed relative to each other, as although insulin response may be identical in two individuals, if insulin sensitivity is not the same, then glucose tolerance will also differ (18).

As described above (see 3.5), part of this work aimed to investigate the ability of the IL-1β-antagonist IL-1Ra to prevent diabetes progression. In addition to the involvement of the IL-1β/ IL-1Ra system in modulating glucose-induced β-cell survival and function, IL-1Ra has several important implications in obesity. Besides liver and spleen, the white adipose tissue has been identified as major source of IL-1Ra even in the basal state, with a further increase in obese conditions in mice and humans (147). Serum IL-1Ra concentrations are elevated 6.5-fold in obese individuals and are positively correlated with leptin levels and to a even higher extent with lean body mass (LBM) and insulin resistance index (IRI) compared to age- and sex-matched lean controls (148). Leptin is a cytokine mainly produced by adipocytes (‘adipocytokine’) that functions as a satiety signal regulating both appetite and weight in rodents and humans (149). Its action on the hypothalamus suppresses food intake and stimulates energy expenditure, therefore playing a major role in the control of body fat stores. A hallmark of most cases of obesity is leptin resistance rather than deficiency. There are several findings suggesting that leptin acts through IL-1β / IL-1RI signaling and that IL-1Ra

may contribute to central leptin resistance in morbid obesity (150). IL-1β has been implicated in mediating leptin-induced reduced food intake, as leptin fails to suppress food intake in IL-1RI$^{-/-}$ mice. Also, IL-1Ra has been shown to counteract leptin-induced reduced food intake, supporting the involvement of the IL-1β/ IL-1Ra network in regulating energy homeostasis. In addition, IL-1Ra$^{-/-}$ mice have a lean phenotype (151) in parallel to an increased food intake relative to their body weight (152) which could result from increased energy expenditure and from a six-fold reduction in circulating leptin concentrations.
A part of this work further investigated the role of IL-1Ra in preventing T2DM in an animal model of obesity and elucidate the importance of adipo-insular interactions mediated by adipocyte-derived factors.

3.7 Signaling Pathways and their Role in β-Cell Function

The mammalian insulin gene is exclusively expressed in β-cells of the endocrine pancreas. It encodes a highly conserved single chain precursor pre-proinsulin (here referred to as insulin gene for simplicity), whose processing produces mature insulin consisting of two polypeptide chains joined by disulphide bonds. In most species, insulin exists as single gene, whereas in mice and rats, two non-allelic insulin genes are present. The human gene is homologous to the rodent insulin II, both contain three exons and two introns, while rodent insulin I lacks the second intron. Tissue specificity and correct initiation of transcription together with physiological regulation are controlled by 5' flanking regions within ~400 bp from the transcription start site that contain cis-acting regulatory elements (153). Precise regulation of transcription is achieved by the interaction of sequence motifs in the promoter with a number of ubiquitous and islet specific transcription factors. This provides a mechanism for combinatorial control whose outcome not only depends on the specific combinations but also on the concentrations of the factors. It results in the formation of complexes that not only can exert positive or negative regulation but can also integrate multiple regulatory inputs into one single output, therefore translating different physiological stimuli into the respective state of insulin gene transcription (153). Amongst the regulatory sequences, E, A and C elements are major determinants.

E boxes bind proteins of the basis helix-loop-helix family (bHLH) which form heterodimers between ubiquitous and cell-restricted members (154). In β-cells, the ubiquitous E47 binds to NeuroD/BETA2, that also plays a role in islet development. A boxes contain a conserved sequence that binds factors belonging to the homeodomain-containing family, of which PDX-1 (pancreatic duodenum homeobox factor-1) is the most important (155; 156). It functions in the early commitment of the primitive gut to pancreatic fate and in

the maturation of β-cells (157) and is a major transactivator of the insulin gene as well as of glucosetransporter-2 (Glut2) and glucokinase (GK). The C1 element has been shown to bind RIPE3b1, which has recently been identified as the bZIP (basic leucine zipper) protein MafA (158). It has been shown that there are cooperative interactions between these proteins that also involves participation of coactivators such as p300/CBP histone acetyltransferases leading to appropriate transcriptional regulation. Of these factors associated with glucose-regulated insulin gene transcription, PDX-1 is the most studied. Glucose promotes the DNA-binding activity (159) as well as transactivation potency of PDX-1 (160). However, there are controversial studies on the signal transduction mechanisms leading to PDX-1 mediated insulin transcription (reviewed in (161)). There is evidence that glucose stimulates modification and translocation of a cytoplasmic, 31 kDa to the nuclear, 46 kDa form. Even though this process requires phosphorylation, the change in molecular mass suggests an involvment of other post-translational modifications such as sumoylation (162). In addition, several kinases were proposed to phosphorylate PDX-1, including p38 mitogen-activated protein kinase, phosphatidylinositol-3 kinase (PI3-K) and extracellular signal-regulated kinases (ERK) -1/2. Despite these controversial findings, the importance of PDX-1 in mediating glucose responsiveness has been underscored by several studies (153). For example, Leibowitz et al. (163) demonstrated that isolated islets from diabetic *psammomys obesus*, which do not respond to elevated glucose, are devoid of the pdx-1 gene product. Interestingly, pdx-1 gene transfer normalized the defect in glucose-stimulated gene expression. Also, MODY4 (maturity onset diabetes of the young, another form of diabetes) is caused by a mutation in PDX-1 (164). In addition, chronically elevated glucose levels *in vitro* impair insulin gene expression which is associated with diminished binding activity of PDX-1 (38). Furthermore, recent studies suggest an important influence of post-translational modifications of PDX-1 in β-cell function under diabetic conditions since oxidative stress (165) as well as the combination of palmitate and elevated glucose (166) induce nucleo-cytoplasmic translocation of PDX-1. These findings emphasize the importance of the regulation of the unique transcriptional complexes in β-cells which assures an appropriate insulin transcription in response to a variety of physiological stimuli such as nutrients (glucose, NEFAs) and hormonal stimuli (insulin, glucagon, leptin, glucagon like peptide-1).

Despite all the signals that affect β-cell function and survival, glucose is the most important fuel and regulator. Besides its well-known role as stimulator of insulin secretion, it also promotes translation to new protein and enhance posttranslational pro-insulin processing. In addition, it is believed to enhance insulin gene transcription and stabilize mature insulin mRNA (167-170), two aspects that have been difficult to investigate due to the very high abundance and long half-life of insulin mRNA. An elegant study by Evans-Molina et al. (171) evaluated the quantities and abundance of insulin mRNA and different species of

pre-mRNAs as well as their changes in response to glucose. They found that only the intron-2 containing pre-mRNA species have a short-enough half-life and a low-enough abundance to reflect acute glucose-induced changes in insulin gene transcription. Already after 30 minutes, they observed glucose-induced increase in H4 acetylation and RNA polymerase II recruitment. Measurable increases of the intron-2 containing pre-mRNAs were delayed by 60 minutes and a rise of mature mRNA levels was not detected before 48 hours. These data further elucidated insulin gene transcription in human islets and emphasizes the necessity of measuring pre-mRNA species to detect acute changes of insulin transcription.

Downstream in glucose-activated pathways, the mitogen-activated protein kinases (MAPKs) ERK1/2 have been implicated as crucial steps in signal transduction that integrate short- as well as long-term nutrient sensing information (172). ERK1/2-activity has been found to correlate with the secretory demand on β-cells as well as insulin secretion. However, the mode of activation is complex and depends specifically on the individual ligands, leading to a precise output signal that incorporates the different stimuli. ERK1/2 action has been shown to regulate different aspects of β-cell function, the most prominent of which is insulin gene transcription. The insulin promoter is positively and negatively regulated by glucose (153) and both require ERK1/2-activity. Acute glucose stimulates insulin gene expression which is dependent on ERK1/2 as demonstrated by inhibition of this effect by the MEK1/2-inhibitors PD098059 or U0126 (173; 174). There are several substrates whose DNA-binding to the insulin promoter requires ERK1/2-activity, including BETA2, MafA and PDX-1. In addition, ERK1/2 might also mediate the deleterious long-term effects of glucose by modulating transcription factors such as NFAT (nuclear factor of activated T-cells) or C/EBP-β (CCAAT/enhancer binding protein-β) that leads to repression of the insulin promoter (174). Moreover, prolonged high glucose-induced impaired function and apoptosis were prevented by inhibiting chronic ERK1/2-activation (175).

The second project in this work aimed to investigate the effects of hyperglycemia on ERK1/2 in regard to their response to an acute glucose challenge. We tested the hypothesis that insulin secretion correlates with ERK1/2 phosphorylation and that both events are diminished after chronic incubations in elevated glucose. Furthermore, we propose that inducing β-cell rest during the chronic period by either inhibiting insulin secretion or ERK1/2-activity restores acute β-cell function and therefore might be a therapeutic strategy to preserve β-cell function in diabetes.

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4. THE POTENT ROLE OF IL-1RA TO PREVENT DIET-INDUCED DIABETES

The first part of this study (Figure 1-7) has been published:
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4.1 SUMMARY

Sub-clinical inflammation is a recently discovered phenomenon in type 2 diabetes (T2DM). Elevated cytokines impair β-cell function and survival. A recent clinical trial shows that blocking Interleukin-1β (IL-1β) signaling by Interleukin-1 Receptor Antagonist (IL-1Ra) improves β-cell secretory function in patients with T2DM. In the present study we provide further mechanisms of the protective role of IL-1Ra on the β-cell.

IL-1Ra improved glucose tolerance and insulin secretion *in vivo* in C57BL/6J mice fed a high fat/ high sucrose diet (HFD) for 12 weeks. High fat diet treatment increased serum levels of free fatty acids, triglycerides and cholesterol and of the adipokines resistin and leptin, all of which were reduced by IL-1Ra. In addition, IL-1Ra counteracted the high fat diet induced decrease in adiponectin.

Studies on isolated islets revealed that IL-1Ra specifically acted on the β-cell. IL-1Ra protected from β-cell apoptosis, induced β-cell proliferation and improved glucose-stimulated insulin secretion. Insulin mRNA was reduced in islets from mice fed a HFD, but normalized in the IL-1Ra group whereas PDX-1 mRNA levels were oppositely regulated, increased by HFD and normalized by IL-1Ra treatment. PDX-1 protein was localized in the cytosol in pancretic sections of HFD animals, but remained in the nucleus in islets when HFD mice were treated with IL-1Ra. Importantly, IL-1Ra counteracted glucose-induced PDX-1 nuclear export in isolated human islets. We found the JNK-pathway as a possible link in the downstream signaling from IL-1Ra to PDX-1. In addition to the diet-induced model of diabetes, we investigated the effect of IL-1Ra in another model of genetically induced T2DM, the db/db mouse. Also in this system, IL-1Ra was found to have a protective role as it was able to delay the onset of diabetes.

Our results show that IL-1Ra improves β-cell survival and function and support the potential role for IL-1Ra in the treatment of diabetes.

4.2 INTRODUCTION

Obesity is a risk factor for insulin resistance and type 2 diabetes (T2DM). Most obese individuals are insulin resistant but compensate by increasing insulin secretion to maintain normoglycemia. The factors determining the amount of insulin that can be secreted are β-cell function as well as β-cell mass. T2DM manifests when the β-cell fails to adaptively increase insulin secretion as a result of impaired β-cell function (2-5), as well as decreased β-cell mass (6-8). The decrease in β-cell mass in both type 1 diabetes (T1DM) and T2DM can be attributed to the increase in frequency of β-cell apoptosis (6; 9; 10). Once hyperglycemia is present, the loss of β-cells accelerates, accompanied by further impairment of β-cell secretory function, both factors in the development of T2DM (11).

The mechanisms of β-cell destruction in a diabetic milieu are complex. Studies on isolated islets show the interplay of glucotoxicity and lipotoxicity (12). Additionally, the β-cells are particularly prone to oxidative stress due to their low expression of antioxidant molecules. Reactive oxygen species cause direct cellular damage in the β-cell by oxidizing nucleic acids and proteins (13) and inactivating genes that are involved in cellular defense (14; 15). Furthermore, the β-cell is especially sensitive to inflammatory attack and in vitro studies have shown that pro-inflammatory cytokines induce β-cell apoptosis and impair function (16).

In human islets, we have provided evidence that glucose-induced β-cell apoptosis and dysfunction are partly mediated by β-cell production and secretion of the pro-inflammatory cytokine Interleukin-1β (IL-1β) (17). After chronic exposure to high glucose, the β-cell itself produces IL-1β, followed by NF-κB activation, Fas up-regulation, DNA-fragmentation and impaired β-cell function (17). IL-1β has been shown to contribute to β-cell destruction in T1DM (reviewed in (18; 19)) as well as in T2DM (reviewed in (11)). Also in three animal models, the Psammomys obesus (17), the GK rat (20) and the human islet amyloid polypeptide transgenic rat (21), (Peter Butler, personal communication), pancreatic β-cell expression of IL-1β under hyperglycemic conditions has been observed. IL-1β signal transduction is initiated by ligand binding to type 1 IL-1 receptor (IL-1R1), allowing docking of the IL-1R accessory protein (IL-1AcP) (reviewed in (22)). This activates downstream effectors, which regulate β-cell survival and function (reviewed in (19; 23)). A promising target to block these deleterious effects of IL-1β (24; 25) as well as of elevated glucose levels (17) is the use of IL-1Ra. IL-1Ra is an anti-inflammatory cytokine and naturally occurring antagonist of IL-1α and β (26-28). Four forms of IL-1Ra have been described, three of them are intracellular proteins (icIL-1Ra I, II and III) and one is secreted (sIL-1Ra) (29). Similarly to IL-1β, IL-1Ra binds to type 1 and 2 IL-1 receptor but lacks a second binding domain. Therefore, IL-1Ra does not recruit IL-1AcP, the second component of the receptor complex. Endogenous production and secretion of sIL-1Ra has been shown to limit inflammation and

tissue damage, but the biological effects of icIL-1Ra remain unclear. Exogenous sIL-1Ra has been reported to protect against IL-1β induced β-cell damage (24; 25), and to counteract both low dose streptozotocin induced diabetes (30) and autoimmune diabetes (31) as well as promoting graft survival (31-33). Also, IL-1Ra protected from glucose as well as IL-1β induced apoptosis in human islets (17). Therefore, the balance of IL-1β and IL-1Ra may play a crucial role in the pathogenesis of diabetes. We have recently shown that IL-1Ra is secreted from β-cells and is expressed in β-cell granules (34). Inhibition of endogenous IL-1Ra by culturing the islets with small interfering RNAs to IL-1Ra, or with leptin for the long term, leads to β-cell apoptosis and impaired function, providing a link from obesity to diabetes (34). The possible crucial role of inflammatory cytokines in the pathogenesis of T2DM is underscored by several recent studies (35). Spranger et al. observed that individuals with elevated levels of IL-1β and IL-6 have a significantly increased risk to develop T2DM (36), pointing to a possible role for IL-1Ra in the treatment of diabetes. Results from a recent clinical study in patients with T2DM showed that IL-1Ra improved glycaemic control and β-cell function (37). In the present study we show that IL-1Ra is able to protect from diabetes progression induced by a high fat diet.

4.3 EXPERIMENTAL PROCEDURES

Animals. For IL-1Ra- or vehicle injections, C57BL/6J wildtype mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 4 weeks of age. One of the 4 following treatments was initiated in mice at 5 weeks of age: Two groups were kept under normal diet (ND, Harlan Teklad Rodent Diet 8604, containing 12.2, 57.6 and 30.2% calories from fat, carbohydrate and protein, respectively). Another two groups were fed a high fat/ high sucrose diet (HFD, "Surwit" Research Diets, New Brunswick, NJ, containing 58, 26 and 16% calories from fat, carbohydrate and protein, respectively (38)). Half of the animals from each diet group were daily treated with IL-1Ra (ND + IL-1Ra and HFD + IL-1Ra; Kineret®, Amgen, Thousand Oaks, CA, i.p. injection at a dose of 10 mg/kg body weight) or with vehicle as control (control ND and control HFD). Throughout the study period of 12 weeks, food consumption and body weight were measured weekly. Four independent experiments with a total of 16 mice (four mice/ cage) in each group were performed.

Transgenic mice overexpressing IL-1Ra (IL-1Ra-OE) were kindly provided by Dr. Emmet Hirsch (Northwestern University, Evanston, IL, USA, (39)). Beginning at 5 weeks of age and continuing for 16 weeks, transgenic animals as well as their wildtype littermates were fed a normal diet (WT ND and IL-1Ra-OE ND) or a high fat/ high sucrose diet (WT HFD and IL-1Ra-OE HFD) as described above. Body weight and food intake was measured weekly. Four independent experiments with a total of 16 mice per group, respectively, were performed.

Heterozygous leptin receptor deficient mice on the C57BLKS/J background ($Lepr^{db/+}$, db/+) were purchased from Jackson Laboratory. By crossbreeding these mice to C57BL/6J-IL-1Ra-overexpressing mice (OE), we obtained diabetic $Lepr^{db/db}$ (db/db) as well as non-diabetic $Lepr^{+/+}$ (WT) with endogenous overexpression of IL-1Ra (OE-db/db and OE) as well as littermates without IL-1Ra as appropriate negativ controls (db/db and WT) with the same mixed background.

All animals were housed in a temperature-controlled room with a 12-hour light/dark cycle and were allowed free access to food and water according to the protocol approved by the UCLA Chancellor's Animal Research Committee in agreement to NIH animal care guidelines.

Intra-peritoneal glucose and insulin tolerance tests. After 4, 8 and 12 weeks of diet and IL-1Ra treatment, all animals underwent *in vivo* studies. For intraperitoneal glucose tolerance tests (IPGTT), mice were fasted 12 hours overnight and injected intraperitoneally with glucose (40%, Phoenix Pharmaceuticals Inc., St. Josephs, MO) at a dose of 2 g/kg body weight. Blood samples were obtained at time-points 0, 15, 30, 60, 90 and 120 min. for glucose measurements using a Glucometer (Freestyle, TheraSense Inc, Alameda, CA) and

at time-points 0 and 30 min. for measurement of serum insulin levels. For intraperitoneal insulin tolerance tests (IPITT), mice were injected intraperitoneally with 0.75 U/kg body weight recombinant human insulin (Novolin, Novo Nordisk) after 5 hour fasting and glucose concentration was determined with the Glucometer.

Islet isolation and culture. After 12 weeks of diet and treatment, mice were sacrificed, blood was taken by cardiac puncture and serum stored at -80°C until further analysis. Thereafter, islets from all groups were isolated as described previously (40). In brief, pancreata were perifused with a collagenase solution (Collagenase type 4, Worthington, Lakewood, NJ, according to the manufacturer's instructions) and digested in the same solution at 37°C followed by washing and handpicking. The islets were then cultured in RPMI-1640 medium containing 11.1 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS (Invitrogen). Human islets were isolated from pancreata of healthy organ donors at the University of Illinois at Chicago as described previously (41) and cultured in CMRL-1066 medium containing 5.5 mM glucose 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FCS. All islets were kept in a humid environment containing 5% CO_2 at 37 ºC. Islets were kept for 24 hours in culture medium in suspension dishes prior to treatment start or to harvesting for islet sections or RNA extraction. For treatment of human and mouse islets, medium was changed to the respective culture medium containing 5.5, 11.1, 22.2 or 33.3 mM glucose or 5.5 mM plus 2 ng/ml recombinant human IL-1β (R&D Systems, Minneapolis, MN), with or without 500 ng/ml recombinant human IL-1Ra (R&D) or 10 µM JNKi (kindly provided by Xigen S.A., Lausanne, Switzerland) for 72 hours.

Glucose stimulated insulin secretion. Islets used to perform glucose-stimulated insulin secretion experiments were kept in culture medium on matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel) for 4 days, allowing the cells to attach to the dishes and spread (42). These experimental conditions allowed direct comparison to our previous studies in human islets pre-treated with IL-1Ra *in vitro* (17). For acute insulin release in response to glucose, islets were washed and pre-incubated (30 min) in Kreb's Ringer bicarbonate buffer (KRB) containing 2.8 mM glucose and 0.5% BSA. KRB was then replaced by KRB 2.8 mM glucose for 1 h (basal), followed by an additional 1 h in KRB 16.7 mM glucose (stimulated). Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Islet insulin was determined using mouse insulin ELISA (ALPCO, Salem, NH).

Serum analysis. Serum obtained by cardiac puncture at the time of sacrifice was analyzed for serum lipids by the UCLA Lipid and Lipoprotein Laboratory as described (43). All lipid

assays were performed in triplicate determinations. An external control sample with known analyte concentration was run for each assay to assure accuracy. Free plasma glycerol concentrations were also determined and used to correct the triglyceride values. Leptin and resistin were measured using a mouse serum adipokine panel (LINCOplex, LINCO). Serum adiponectin and insulin concentrations were determined using mouse adiponectin and insulin ultrasensitive ELISA (ALPCO). Serum IL-1Ra levels were evaluated using mouse IL-1Ra Quantikine ELISA (R & D, Minneapolis, MN, USA).

Pancreatic insulin and glucagon content. To determine the total pancreatic insulin and glucagon content, ~30 mg of pancreatic tissue from eight mice/ treatment group, respectively was homogenized in 1 ml 0.18 N HCl in 70% ethanol and incubated overnight at 4°C. After centrifugation, supernatants were collected and stored at -80°C. Insulin concentrations were measured using mouse insulin ELISA (ALPCO) and glucagon levels were determined by glucagon EIA (ALPCO) and values were normalized to tissue weight.

Adipocyte size. Epididymal fat pads were dissected and fixed overnight at 4°C in 4% paraformaldehyde, followed by washing in 30% sucrose/ PBS for 12 hours at 4°C. After 30 minutes incubation in a 1:1 mixture of 30% sucrose and OCT (Tissue Tek, Sakura Finetek, CA), the tissue was placed in 100% OCT for 30 minutes, embedded in plastic molds and frozen on dry ice before sectioning. To determine adipocyte size, sections were stained with hematoxylin and eosin. Cross-sectional adipocyte area was measured by manual tracing of 300 or more cells per mouse and 6 animals per treatment group using an Olympus IX70 inverted system microscope (Olympus America, Melville, NY) and Image-Pro Plus software (Media Cybernetics, Silver Springs, MD).

β-cell mass and histochemical analyses. After 12 weeks of diet and treatment, pancreata were weighed and fixed overnight in 4% paraformaldehyde at 4°C under continuous shaking followed by paraffin embedding, orienting pancreata such that sections were cut along the head-tail axis. To obtain sections from isolated mouse or human islets, islets were washed with PBS, fixed in Bouin's solution for 15 minutes and resuspended in 2% melted agarose in PBS, followed by short centrifugation and paraffin embedding. To determine β- and α-cell mass, ten sections (spanning the width of the pancreas) were deparaffinized, rehydrated and incubated overnight at 4°C with guinea-pig anti-insulin antibody (Dako, Carpinteria, CA), followed by detection with a fluorescein-conjugated donkey anti-guinea pig antibody (Dako). Subsequently, the specimens were labeled for glucagon with rabbit anti-glucagon (Dako), followed by detection with donkey anti-rabbit Cy3-conjugated antibody (Dako). An image of each slide was captured using Openlab and ImageJ software (Improvision Inc., Lexington,

MA). Tissue areas were determined by marking the image for total tissue and for β-cells (fluorescein-labeled) or α-cells (Cy3-labeled), respectively. β- and α-cell mass was analyzed using Openlab software. The relative area of β-cells (green fluorescence) or α-cells (red fluorescence) was determined by quantification of the cross-sectional β- or α-cell area divided by the cross-sectional area of total tissue, respectively. The cell mass per pancreas was estimated as the product of the relative cross-sectional area of β-cells or α-cells per total tissue and the weight of the pancreas. For analysis of β-cell proliferation, mouse pancreas sections were deparaffinized, rehydrated and incubated overnight at 4°C with rat anti-mouse Ki67 (Dako), followed by detection with donkey-anti-rat Cy3-conjugated antibody (Dako). For detection of β-cell apoptosis, sections were incubated with 20 κ g/ml proteinase K (Roche Diagnostics, Indianapolis, IN) for 15 minutes at 37°C and apoptosis was analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, TMR red; Roche). For PDX-1 localization studies, sections were incubated overnight at 4°C with rabbit anti-PDX-1 (kindly provided by Christopher Wright, Vanderbilt University Medical Center, Nashville, TN) followed by detection with donkey anti-rabbit Cy3-conjugated antibody (Dako). Subsequently, all sections were double-stained for insulin as described above. Same staining was performed on sections of isolated islets. Fluorescent slides were analyzed using Leica DM6000 microscope or Leica DMIRE2 confocal microscope and images aquired using Openlab software.

RNA extraction and quantitative RT-PCR analysis. Total RNA of isolated islets was extracted after overnight culture as described previously (44). Total RNA from epididymal fat pads was isolated using RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA). For quantitative analysis, we used the LightCycler Quantitative PCR System (Roche) with a commercial kit (LightCycler FastStart DNA Master plus SYBR Green I, Roche). Mouse primers used were: 5'-ttcttctacacaccca-3' and 5'-ctagttgcagtagttct-3' (insulin); 5'-gtccatgccatcactgccac-3' and 5'-cagcaccagtggatgcaggg-3' (GAPDH); 5'-gttggccaggctggtgtccag-3' and 5'-ctgtgatgagctgctcagggtgg-3' (tubulin); 5'-ctttggctatgggcttccagtc-3' and 5'-gcaaggaggacagagtttatcgtg-3' (F4/80); 5'-ctggatagcctttcttctgctg-3' and 5'- gcacactgtgtccgaactc-3' (CD11c); 5'-acggcatggatctcaaagac-3' and 5'-agatagcaaatcggctgacg-3' (TNFα), 5'-gaccttccaggatgaggaca-3' and 5'-agctcatatgggtccgacag-3' (IL-1β); 5'-ccagctcattgctgggtact-3' and 5'-cagctgactcaaagctggtg-3' (IL-1Ra); 5'-gaggacccgtactgcctaca-3' and 5'-cggggtcccgctactacgtt-3' (PDX-1).

Western Blot analysis. At the end of the incubation periods, islets were washed in ice-cold PBS and lysed for 40 minutes on ice in 40 μl lysis buffer containing 20 mM Tris acetate, 0.27

M sucrose, 1 mM EDTA, 1 mM EGTA, 50mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate. Prior to use, the lysis buffer was supplemented with Protease- and Phosphatase-inhibitors (Pierce, Rockford, IL, USA). Equivalent amounts of protein from each treatment group were run on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and electrically transferred onto PVDF membranes. Membranes were incubated with rabbit anti-phospho JNK (Thr183/Tyr185) or rabbit anti-β-actin (Cell Signaling Technology) antibodies, followed by horseradish-peroxidase-linked anti-rabbit IgG.

Statistical analysis. Samples were evaluated in a randomized manner by a single investigator (N.S.S.) who was blinded to the treatment conditions. Data are presented as means +/- SE and were analyzed by paired, Student's *t* test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

4.4 RESULTS

4.4.1 IL-1Ra Prevents Diet-Induced Diabetes

IL-1Ra has no impact on weight gain or food intake in mice.

To assess the effect of IL-1Ra on diet-induced diabetes, we injected C57BL/6J mice daily with IL-1Ra or with vehicle for 12 weeks. Mice were fed a normal (ND) or a high fat/ high sucrose diet (HFD). Mice fed the HFD gained more weight than the ND control group; this was not influenced by IL-1Ra treatment. From one week of diet and treatment onward, body weight was significantly increased in mice fed the HFD. After 12 weeks, body weight of control mice was 35.6 ± 1.7 g at HFD compared to 24.1 ± 0.5 g at ND, $p<0.001$, and of IL-1Ra-treated mice 34.3 ± 1.3 g at HFD compared to 25.4 ± 0.5 g at ND, $p<0.001$ (Fig.1A). Average weekly food intake was stable throughout the study in both ND- and HFD-groups and was not changed by IL-1Ra-treatment (Fig.1B). Similar results were obtained from transgenic mice overexpressing IL-1Ra (IL-1Ra-OE, data not shown).

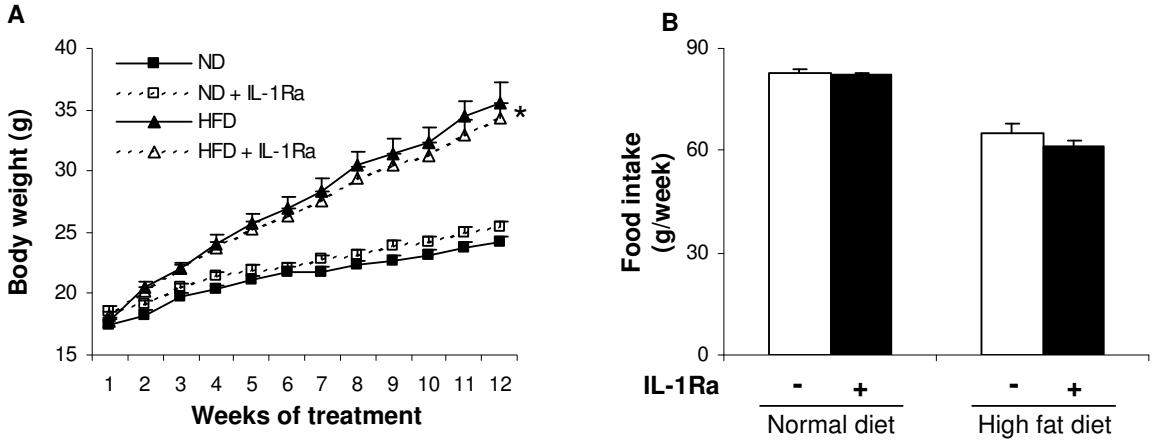

Figure 1: IL-1Ra has no impact on weight gain or food intake in mice.
C57BL/6J wildtype mice were fed a normal (ND) or a high fat/ high sucrose diet (HFD) for 12 weeks. Mice were daily i.p. injected with 10 mg/kg body weight recombinant IL-1Ra (ND + IL-1Ra or HFD + IL-1Ra) or with vehicle (ND or HFD). Body weight **(A)** and food intake **(B)** per cage of four mice was measured weekly. Data show results from a total of 16 animals per group, performed in four separate experiments ± SE. *$p<0.01$ HFD compared to ND same treatment from 1 week on.

IL-1Ra protects from diet-induced hyperglycemia and improves glucose tolerance in mice.

Before and throughout the treatment period, we measured fasting and fed blood glucose every 4 weeks. Average fasted glucose levels before beginning the study in 5-week-old mice were 4 mM glucose and insulin levels 0.4 ug/l insulin, similar as measured in control ND mice throughout the study. As shown in Figure 2, neither fasting (Fig. 2A) nor fed (Fig. 2B) blood

glucose concentrations changed during the 12 weeks of the study in both control and IL-1Ra-treated mice under the normal diet. After 4 weeks of treatment, no differences in glucose levels were observed in all four treatment groups (Fig.2 A,B). After 8 weeks, fasting glucose was 1.9-fold increased in the HFD-group compared to the ND-group. This increase was prevented in animals from the HFD-group that received IL-1Ra (Fig.2A). After 12 weeks, high fat feeding increased both fasting and fed glucose levels (1.7-fold and 1.5-fold increase in fasting and fed glucose levels, respectively, in the HFD-group, compared to ND, Fig.2 A,B, $p<0.001$), whereas in the IL-1Ra-treated HFD-group, glucose levels were significantly lower than in the vehicle HFD-group (1.2-fold reduction compared to the untreated HFD-group in all conditions, $p<0.05$). In parallel, glucose tolerance was impaired in the HFD-group already after 4 weeks (data not shown) and was further declined during the experiment. Fig. 2C shows the response to an intraperitoneal glucose challenge after 12 weeks of diet and treatment. High fat feeding resulted in significantly higher glucose levels before (0 min) and 30, 60, 90 and 120 min after glucose injection ($p<0.05$). IL-1Ra administration protected the HFD-mice from this effect, resulting in blood glucose levels that were significantly lower compared to controls at all time points during the IPGTT ($p<0.05$). Moreover, IL-1Ra treatment led to decreased peak glucose levels in the normal diet group compared to control animals, which reached significance at 15 min (1.3-fold reduction, $p<0.001$, Fig.2C).

Fig. 1D shows serum insulin levels during an IPGTT after 12 weeks of diet and treatment. As previously described (45), high fat feeding resulted in hyperglycemia (Fig.2B,C) as well as hyperinsulinemia (Fig.2D) compared to control diet mice. Compared to ND counterparts, fasted insulin levels were 2.9-fold higher in HF fed mice ($p<0.01$). These mice also failed to further increase their insulin levels in response to a glucose challenge. HFD-mice under IL-1Ra treatment exhibited significantly lower fasting insulin levels (1.7-fold, $p<0.01$) and increased serum insulin concentrations during the IPGTT 2.5-fold. Under the normal diet, IL1-Ra treatment resulted in a higher stimulatory index compared to untreated animals (1.9- vs. 4.2-fold, $p<0.05$).

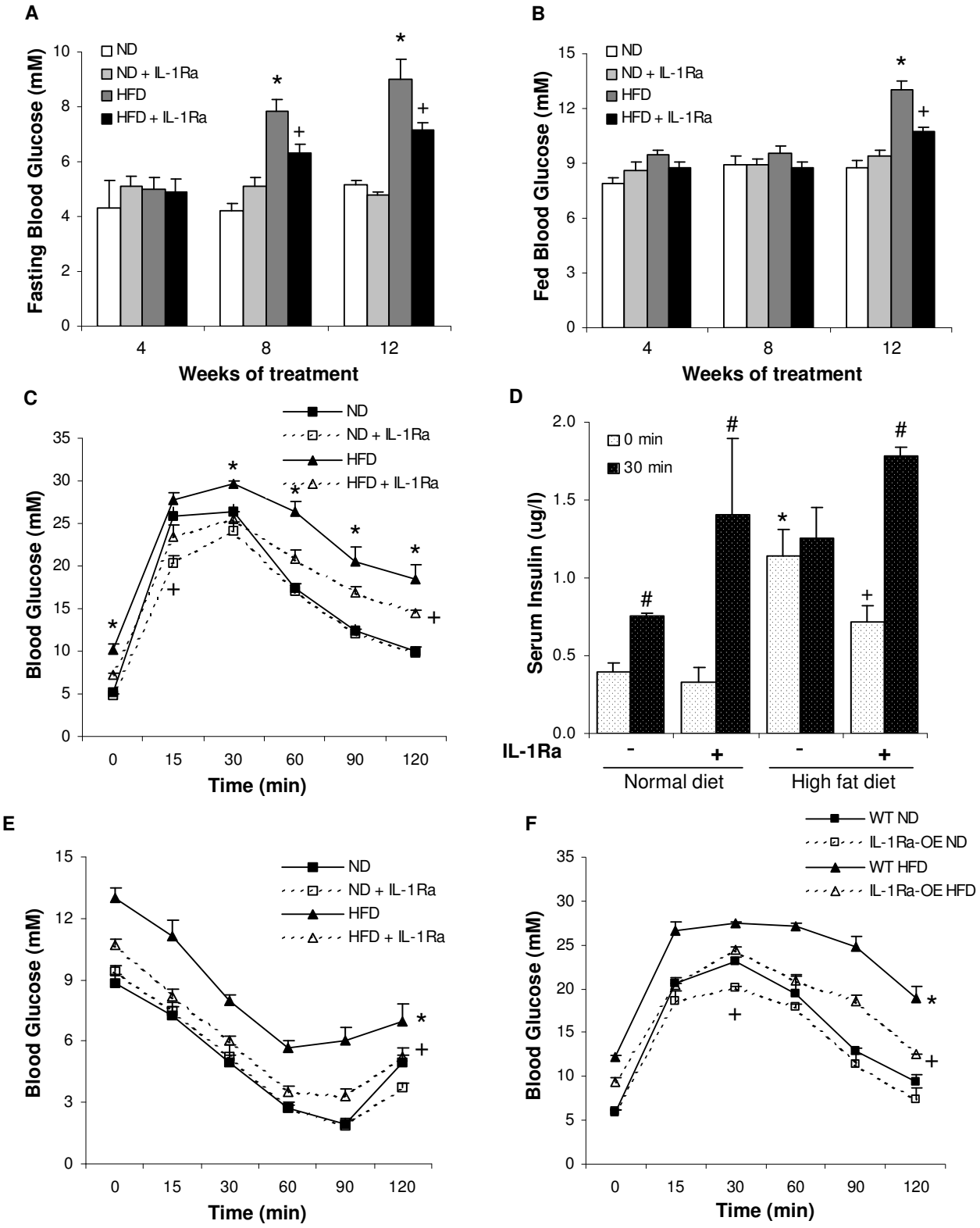

Figure 2: IL-1Ra protects from diet-induced hyperglycemia and improves glucose tolerance.
(A,B) Blood glucose levels were measured after 4, 8 and 12 weeks of diet and IL-1Ra treatment after overnight fasting **(A)** or without fasting **(B)**. Blood glucose **(C)** and plasma insulin **(D)** levels following i.p. injection of 2 g/kg body weight glucose in mice after 12 weeks of treatment. **(E)** Blood glucose concentrations of mice after 12 weeks of treatment, undergoing an insulin tolerance test. Insulin was injected i.p. at a concentration of 0.75 U/kg body weight. **(F)** Blood glucose levels during IPGTT experiment as described above in IL-1Ra-overexpressing (IL-1Ra-OE) animals and their wildtype (WT) littermates after 16 weeks of normal or high fat diet. Data show mean ± SE. $^{*}p<0.05$ HFD compared to ND, $^{+}p<0.05$ IL-1Ra or IL-1Ra-OE compared to vehicle or wildtype same diet, respectively, $^{\#}p<0.05$ stimulated compared to basal insulin secretion. Data were collected from 16 animals per group, performed in four separate experiments.

To determine insulin sensitivity, we performed an insulin tolerance test by measuring glucose concentrations after intraperitoneal insulin injection of 0.75 U/kg of body weight. No significant difference in insulin sensitivity was found among the animals of the normal diet group (Fig.2E). As previously described, untreated HFD-mice displayed impaired insulin sensitivity compared to ND-mice. Animals that received IL-1Ra injections were protected against this high fat diet-induced insulin resistance (Fig.2E).

Similar results were obtained from IL-1Ra-OE mice. After 16 weeks of HFD, glucose tolerance was impaired showing increased glucose levels at all time-points during the IPGTT as well as increased fasting glucose levels in the wildtype mice, compared to ND ($p<0.05$, Fig.2F). This was prevented in IL-1Ra-OE mice under the HFD, showing significantly reduced fasting glucose levels and improved glucose tolerance at all time-points during the IPGTT ($p<0.05$, Fig.2F). Also, IL-1Ra overexpression led to decreased peak glucose levels in the ND-group compared to wildtype mice, which reached significance at 30 min (1.2-fold reduction, $p<0.001$, Fig.2F).

IL-1Ra treatment has no effect on β-cell or α-cell mass.

Pancreatic weight per body weight remained unchanged by diet and treatment (data not shown). Immunohistochemical evaluation of pancreata of all four treatment groups after 12 weeks of diet and treatment revealed a normal islet structure (Fig. 3B). Islet β-cell mass was dramatically increased by high fat feeding in vehicle- and in IL-1Ra-injected animals compared to normal diet (2.3-fold increase in the control and 2.6-fold increase in the IL-1Ra-treated HFD-group compared to ND, respectively, $p<0.001$; Fig. 3C). High fat feeding also resulted in increased α-cell mass (2.1 fold and 1.8-fold increase in the control and the IL-1Ra-treated HFD-group compared to ND, respectively, $p<0.05$, Fig. 3D). IL-1Ra-treatment did not affect β-cell mass nor α-cell mass in both ND and HFD groups. Consistent with an increase in β-cell mass, the pancreas of control and IL-1Ra-treated high fat diet-mice contained 2.6-fold and 3.1-fold more insulin as compared to their normal diet counterparts ($p<0.01$, Fig. 3E). In addition, pancreatic glucagon content also increased by high fat feeding (3.3-fold and 2.6-fold increase in the control and the IL-1Ra-treated HFD-group compared to the respective ND-group, $p<0.05$, Fig. 3F). In consistency with the increase in β-cell mass, we also observed larger islets from both HFD treated groups compared to ND during islet isolation.

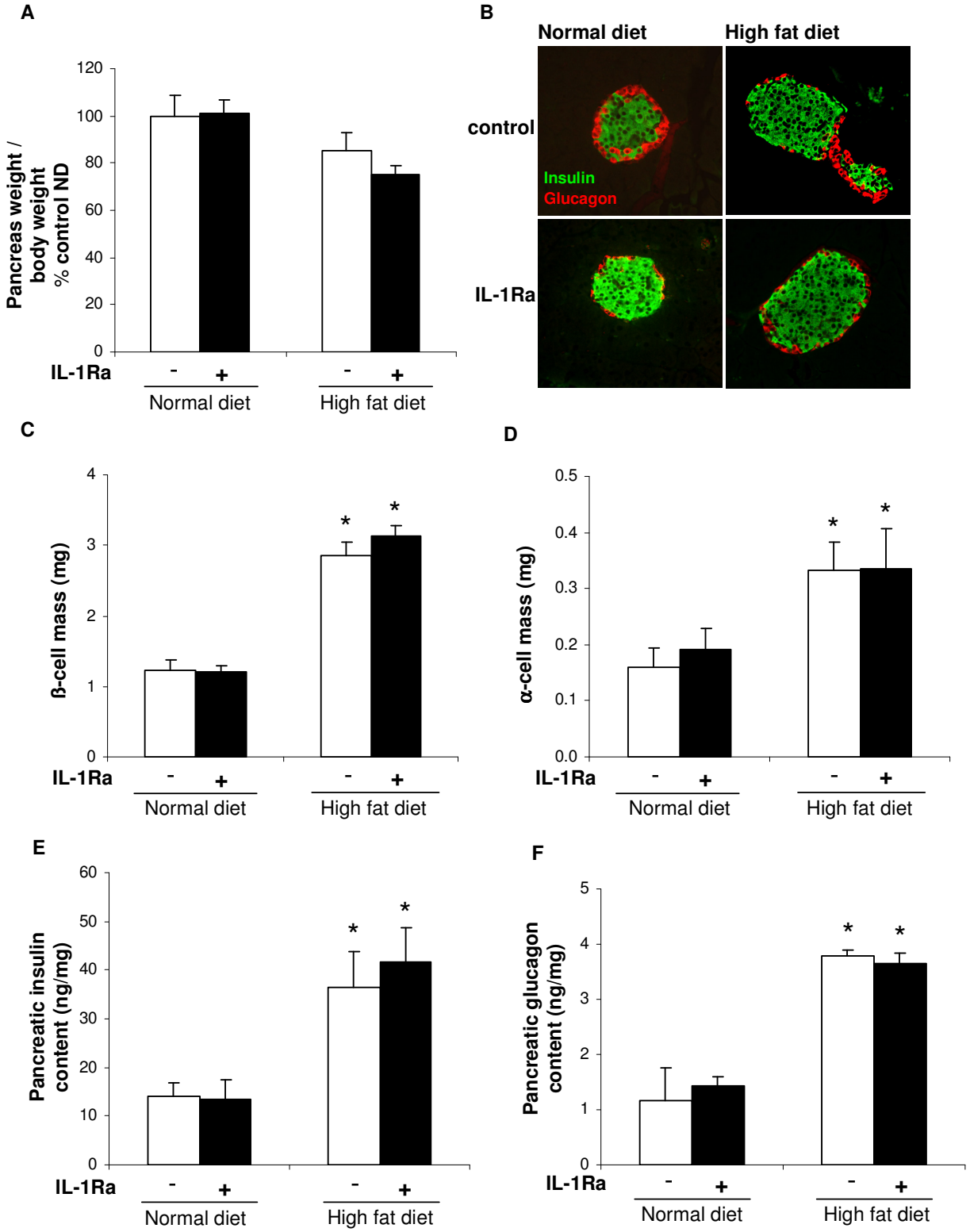

Figure 3: IL-1Ra treatment has no effect on β-cell mass or α-cell mass.
(A) Pancreatic weight per body weight was measured at the end of the study and is expressed relative to control normal diet conditions. **(B)** Double immunostaining for insulin in green and glucagon in red (Magnification x125), **(C)** β-cell mass and **(D)** α-cell mass in tissue sections spanning the width of the whole pancreas of vehicle-treated control mice or IL-1Ra-injected mice fed a normal or high fat diet for 12 weeks (n=4 mice for each group). The β- and α-cell mass per pancreas was estimated as the product of the relative cross-sectional area of β- or α-cells (determined by quantification of the cross-sectional area occupied by β- or α-cells divided by the cross-sectional area of total tissue) and the weight of the pancreas. Pancreatic insulin **(E)** and glucagon **(F)** content in ng per mg pancreas of normal and high fat fed mice injected with vehicle or IL-1Ra for 12 weeks.
*p<0.05 HFD compared to ND same treatment, $^{+}$p<0.05 IL-1Ra-treated compared to vehicle same diet.

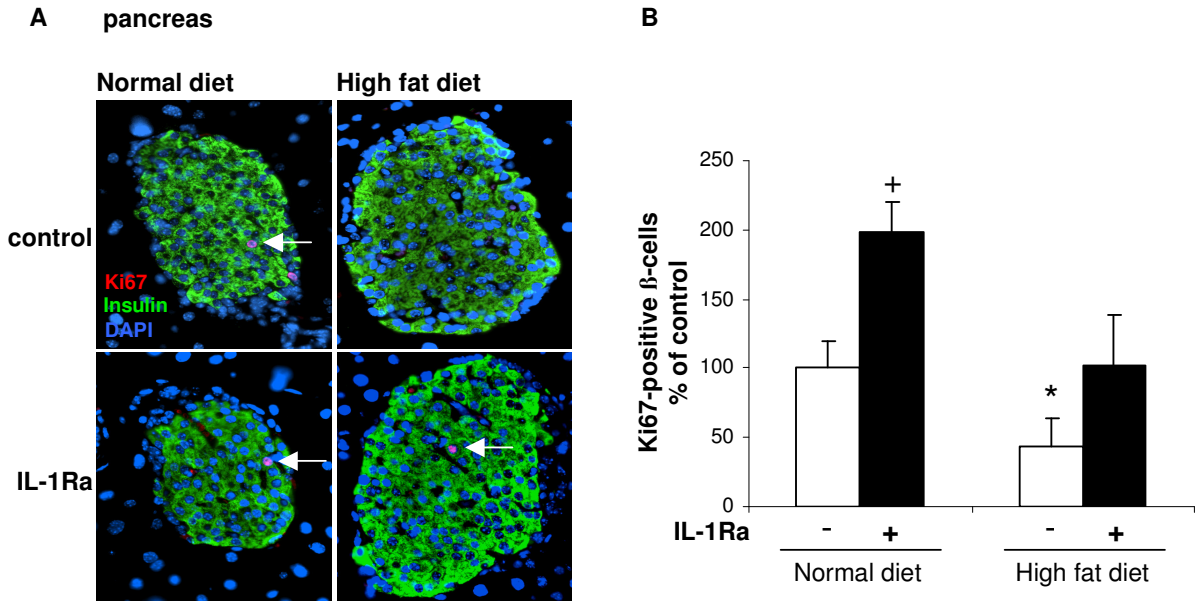

Figure 4: IL-1Ra increases β-cell proliferation in animals under the ND.
(A,B) Triple staining for proliferation by Ki67-antibody in red, insulin in green and DAPI in blue was performed on fixed, paraffin embedded mouse pancreas sections (Magnification x250). **(B)** Results are expressed as percentage of Ki67-positive β-cells ± SE normalised to control ND islets (100%, in absolute values: 0.3 ± 0.1 % Ki67-positive cells in isolated islets from vehicle-treated mice under the normal diet). The mean number of β-cells scored was 4599 ± 643 for each treatment condition in four independent experiments. *p<0.05 HFD compared to ND same treatment, $^{+}$p<0.05 IL-1Ra-treated compared to vehicle same diet.

IL-1Ra induces proliferation after 12 weeks in mice under the normal diet.

In cultured human islets, IL-1Ra increased basal β-cell proliferation (46) and protected from hyperglycemia induced β-cell apoptosis (17). Therefore, we investigated β-cell turnover in islets from all four groups after 12 weeks of diet and treatment. Staining of pancreatic sections for the replication marker Ki67 revealed that IL-1Ra-injections increased β-cell proliferation 2.0-fold (Fig. 4A;B p<0.05) in the ND-mice. In contrast, high fat feeding reduced proliferation 2.2-fold in vehicle-treated animals compared to the control ND-group (Fig. 4A,B), whereas IL-1Ra-treated mice on the HFD were protected against this decrease in proliferation. Same results were obtained from isolated islets. Proliferation was 2.0-fold increased (p<0.05) *in vitro* in islets from IL-1Ra-injected ND-mice. In the HFD-group, islets from IL-1Ra-injected mice were protected against the decreased proliferation observed in control HFD-mice.

IL-1Ra protects from β-cell apoptosis in cultured isolated islets.

In addition to proliferation, we investigated β-cell apoptosis in pancreatic sections. Staining revealed no TUNEL-positive β-cells in sections from both ND-groups and from the IL-1Ra-treated HFD-group. We detected 0.016% TUNEL-positive β-cells/ islet in sections from the vehicle treated HFD-group (1 TUNEL-positive β-cell was found in 61 islets analyzed). Due to this low frequency of positive β-cells in pancreatic sections, we analyzed the occurrence of β-cell apoptosis in isolated islets (Fig. 5A). We found a 3.2-fold increase in β-cell apoptosis in islets derived from the high fat diet group. Islets from high fat fed-mice that were treated with IL-1Ra showed a rate of β-cell apoptosis similar to islets from the normal diet group (Fig 5B).

IL-1Ra treatment preserves β-cell function in high fat fed-mice.

Since we observed a protective effect of IL-1Ra on blood glucose levels, glucose tolerance as well as insulin secretion during the glucose tolerance test, we investigated if this effect can be explained by improved β-cell function. Islets were isolated from all four treatment groups and insulin secretion into the culture medium was measured. Acute glucose-stimulated insulin secretion was completely abolished in islets derived from animals fed a high fat diet (Fig. 5C). We found that islets from high fat fed animals, which were treated with IL-1Ra, showed a significant response to a glucose challenge and a stimulatory index comparable to normal diet animals (Fig. 5D). In parallel, high fat feeding induced a decrease in insulin mRNA in isolated islets, which was prevented by IL-1Ra administration (Fig. 5E).

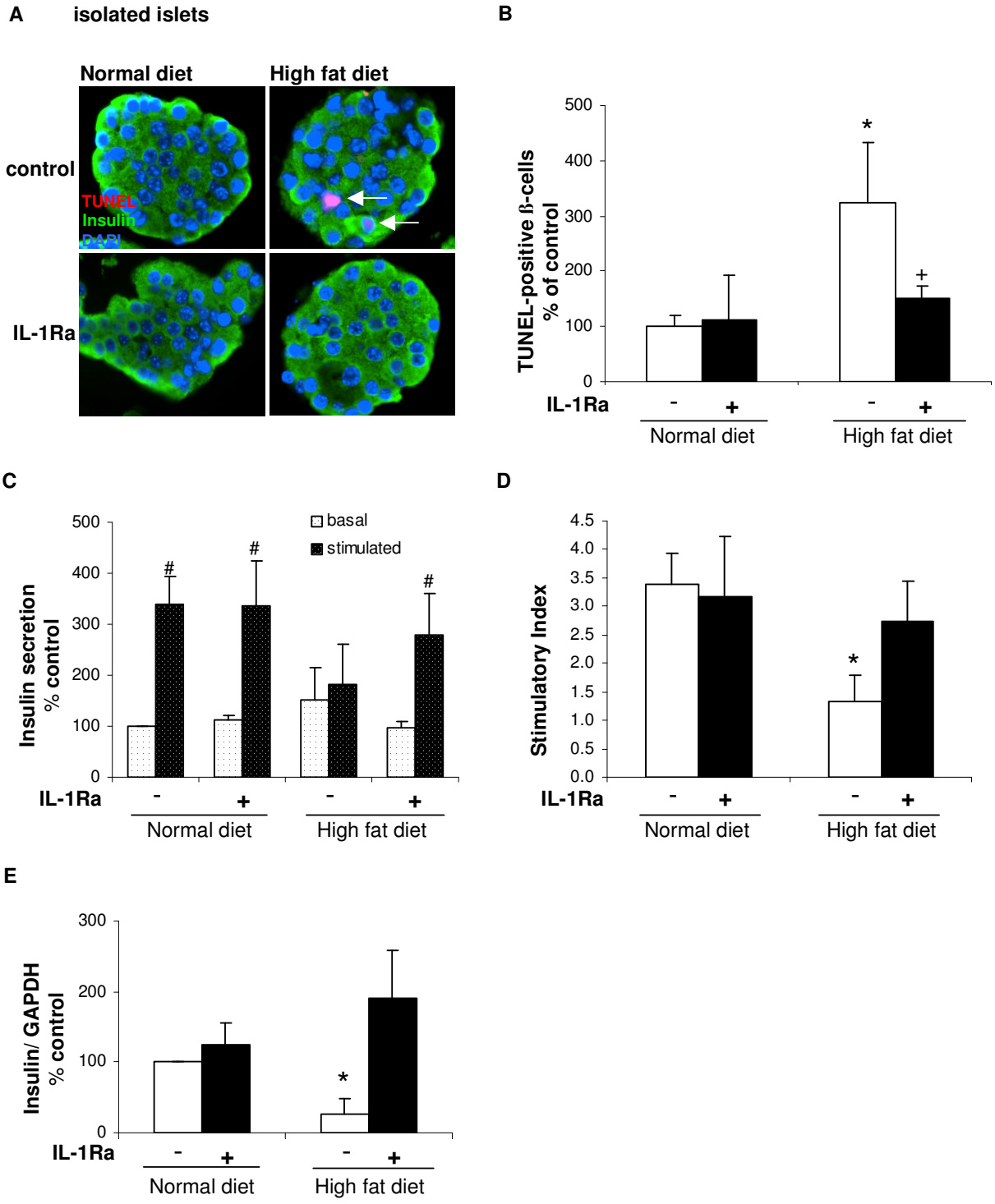

Figure 5: IL-1Ra protects from β-cell apoptosis and preserves β-cell function in cultured isolated islets of HFD-mice. Islets were isolated after 12 weeks of diet and treatment. **(A)** Triple staining for TUNEL in red, insulin in green and DAPI in blue was performed on fixed, paraffin embedded islet sections (Magnification x750). **(B)** Results are expressed as percentage of TUNEL-positive β-cells ± SE normalised to control ND islets (100%, in absolute values: 1.02 ± 0.19 % TUNEL-positive β-cells in isolated islets from vehicle-treated mice under the normal diet). The mean number of β-cells scored was 2169 ± 894 for each treatment condition from six individual mice. **(C,D)** For glucose-stimulated insulin secretion (GSIS) experiments, islets were cultured on extracellular matrix-coated dishes. **(C)** Glucose-stimulated insulin secretion of islets. Basal and stimulated insulin secretion during successive 1-hour incubations at 2.8 mM (basal) and 16.7 mM (stimulated) glucose. **(D)** Stimulatory index denotes the ratio between stimulated and basal values of insulin secretion. Assay was performed in triplicate from four individual mice. **(E)** Quantitative RT-PCR analysis of insulin expression relative to control normal diet conditions. In the LightCycler System the levels of insulin expression were normalized to GAPDH and tubulin with the same result. mRNA levels were evaluated from 6 individual mice per treatment group. Data are shown as mean ± SE. *$p<0.05$ HFD compared to ND same treatment, $^{+}p<0.05$ IL-1Ra-treated compared to vehicle same diet, $^{\#}p<0.05$ stimulated compared to basal insulin secretion.

IL-1Ra prevents HFD-induced changes in serum adipokine and lipid levels.

Epididymal fat pad mass was significantly increased in both HFD-groups (3.7-fold in the control and 3.8-fold in the IL-1Ra-treated HFD-group, compared to ND-group, respectively, $p<0.01$, Fig. 6A). IL-1Ra-injections did not affect epididymal fat mass in both diet groups.

At the end of the study, we measured serum adipokines, lipids and cytokines known to affect insulin action and secretion. IL-1Ra levels are unchanged by the HFD. IL-1Ra concentrations in IL-1Ra-injected mice were increased under both diets (Fig. 6B, $p<0.05$).

High fat diet treatment has been shown to increase serum levels of lipids and of the adipokines resistin and leptin (47). To test the hypothesis that IL-1Ra is able to prevent those changes despite the increase in fat mass, we analyzed serum from all mice for these adipokines.

Levels of leptin and resistin were raised 22-fold and 1.8-fold in the vehicle-treated HFD-group compared to ND, respectively (Fig. 6C;D $p<0.05$). IL-1Ra treatment led to a 3.9-fold and 1.6-fold reduction in leptin and resistin levels in the HFD-group, respectively ($p<0.05$).

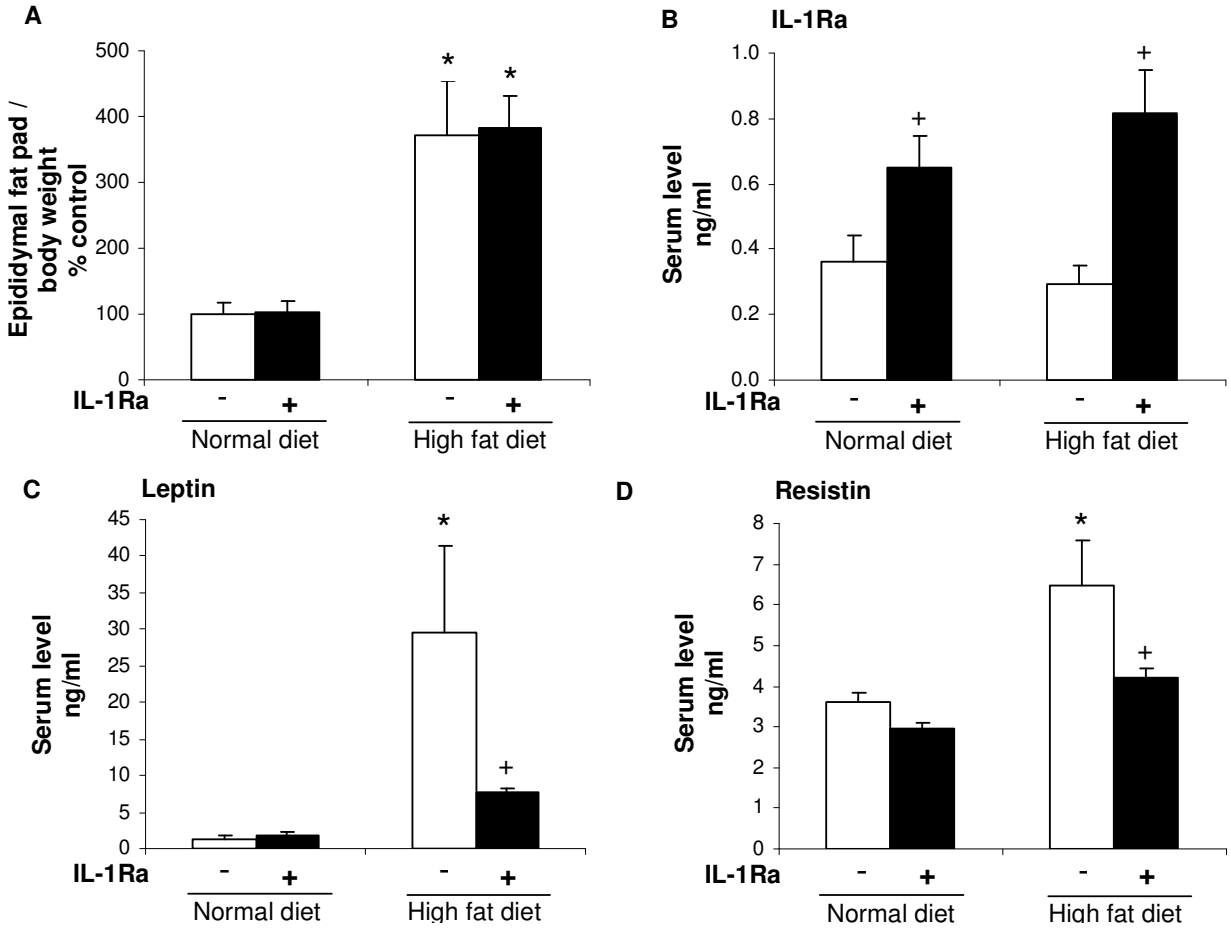

Figure 6: IL-1Ra treatment prevents HFD-induced changes in serum adipokine levels.
(A) Epididymal fat pad weight per body weight was measured at the end of the study and is expressed relative to control normal diet conditions. Serum levels of IL-1Ra **(B)**, leptin **(C)** and resistin **(D)** in mice after 12 weeks of diet and treatment. Data show mean results from 8 mice per treatment group from four independent experiments ± SE. *$p<0.05$ HFD compared to ND, $^{+}p<0.05$ IL-1Ra-treated HFD compared to vehicle HFD.

IL-1Ra prevents HFD-induced changes in serum lipid levels.

Free fatty acid (FFA) levels were increased by 21% in vehicle-treated HFD-animals compared to ND ($p<0.05$, data not shown). In contrast, animals under the HFD that were given IL-1Ra did not show any significant rise in FFA levels compared to their ND-fed counterparts. High fat feeding also significantly increased triglyceride (TG) and cholesterol (Chol) levels (TG: 1.4-fold increase, Chol: 2.1-fold increase, $p<0.05$). IL-1Ra-administration to HFD-mice had the tendency to reduce triglyceride and cholesterol levels compared to vehicle-treated animals, however, this reduction did not reach statistical significance (data not shown). Mice under the high fat diet, which endogenously overexpress IL-1Ra (IL-1Ra-OE), showed significantly reduced levels of FFA as well as triglyceride and cholesterol (Fig. 7A-C, $p<0.05$). Additionally, circulating adiponectin levels were reduced by 20% in the HFD mice compared to ND-mice ($p<0.05$, Fig. 7D). HFD-induced reduction in adiponectin levels was prevented in mice overexpressing IL-1Ra ($p<0.01$, compared to wildtype HFD-mice). In the ND-groups, neither IL-1Ra-injections nor overexpression affected lipid or adipokine levels. Since IL-1Ra-induced changes of adipokine and lipid levels in the HFD-group occurred without changes in fat mass, we investigated the size of adipocytes in hematoxylin- and eosin-stained sections of epididymal fat pads. Measuring the cross-sectional area by manual tracing revealed an over 3-fold high fat diet-induced increase in adipocyte size in both wildtype and IL-1Ra-OE mice compared to their ND counterparts ($p<0.005$, Fig. 7E). IL-1Ra overexpression did not affect adipocyte size in either diet group. To further elucidate the underlying mechanisms of the observed changes in serum adipokines and lipids, we isolated RNA from epididymal fat tissue and assessed expression of inflammatory genes by quantitative RT-PCR. While in wildtype mice high fat diet increased adipose tissue expression of IL-1β, F4/80, CD11c and TNFα ($p<0.05$), this induction was attenuated in IL-1Ra-OE mice ($p<0.05$, Fig. 7F). IL-1Ra-expression in adipocytes was increased by HFD in wildtype animals. In IL-1Ra-OE mice, we confirmed increased IL-1Ra mRNA in adipocytes (6.9-fold increase in IL-1Ra mRNA in ND and 2.7-fold increase in HFD, compared to wildtype mice, $p<0.05$, Fig.7F).

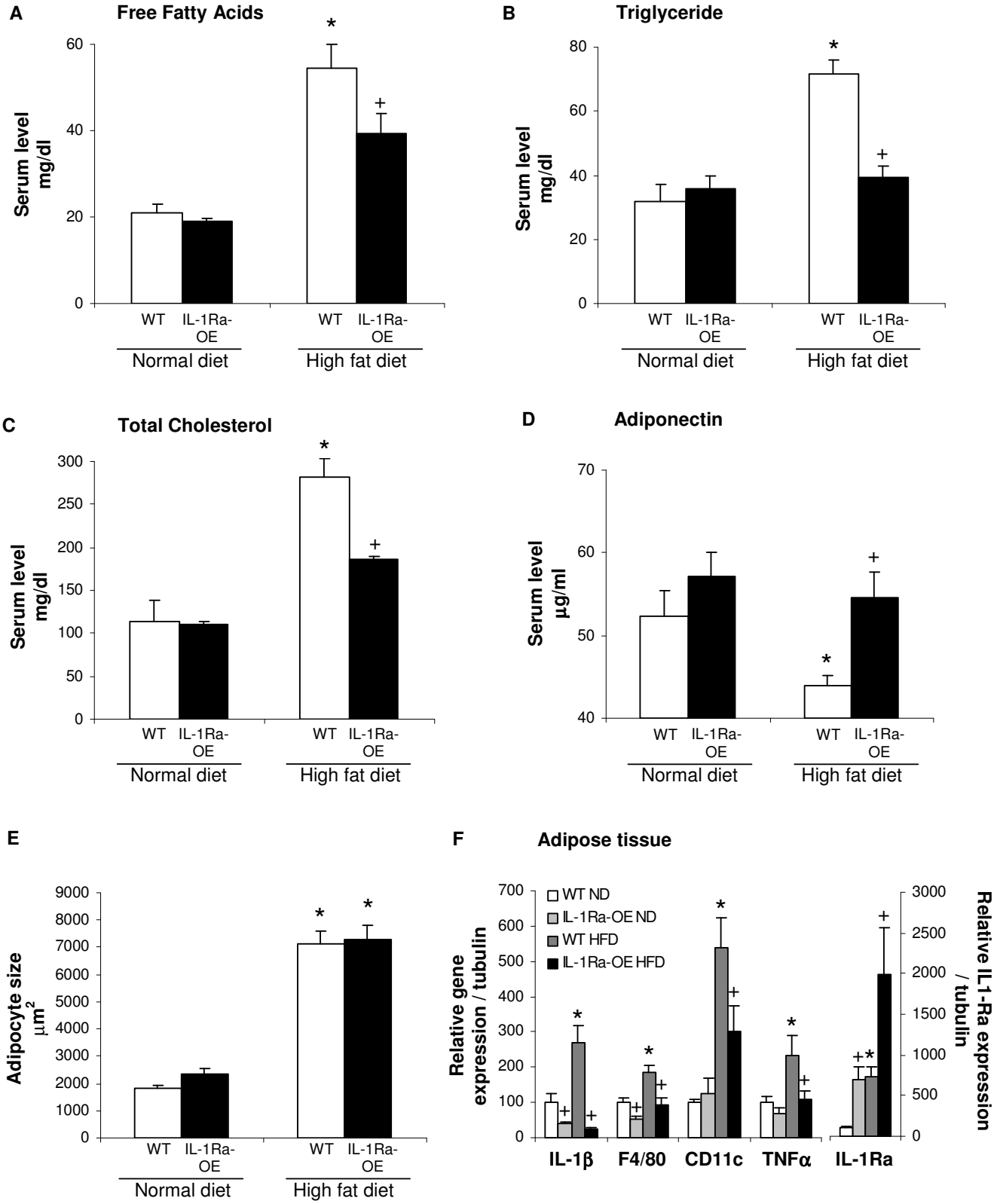

Figure 7: IL-1Ra prevents HFD-induced changes in serum lipid levels.
(A-D) Serum levels of triglycerides, cholesterol, free fatty acids and adiponectin in IL-1Ra-overexpressing (IL-1Ra-OE) mice and their wildtype littermates (WT), fed a normal or high fat diet for 16 weeks (n=8). **(E)** Size of adipocytes in epididymal fat tissue of IL-1Ra-OE mice or WT littermates under ND or HFD. Sections were stained with hematoxylin and eosin followed by measuring cross-sectional area by manual tracing (n=6). **(F)** Quantitative RT-PCR of inflammatory gene expression in epididymal fat pads. Total RNA was isolated after 16 weeks of normal or high fat diet and gene expression was evaluated in the LightCycler System after normalization to tubulin. Results are expressed relative to mRNA levels in wildtype mice under normal diet (n=7). All data are shown as mean ± SE. *p<0.05 HFD compared to ND, +p<0.05 IL-1Ra-OE mice compared to WT same diet. Analyses were performed in four independent experiments.

4.4.2 IL-1Ra Maintains PDX-1 Nuclear Localization

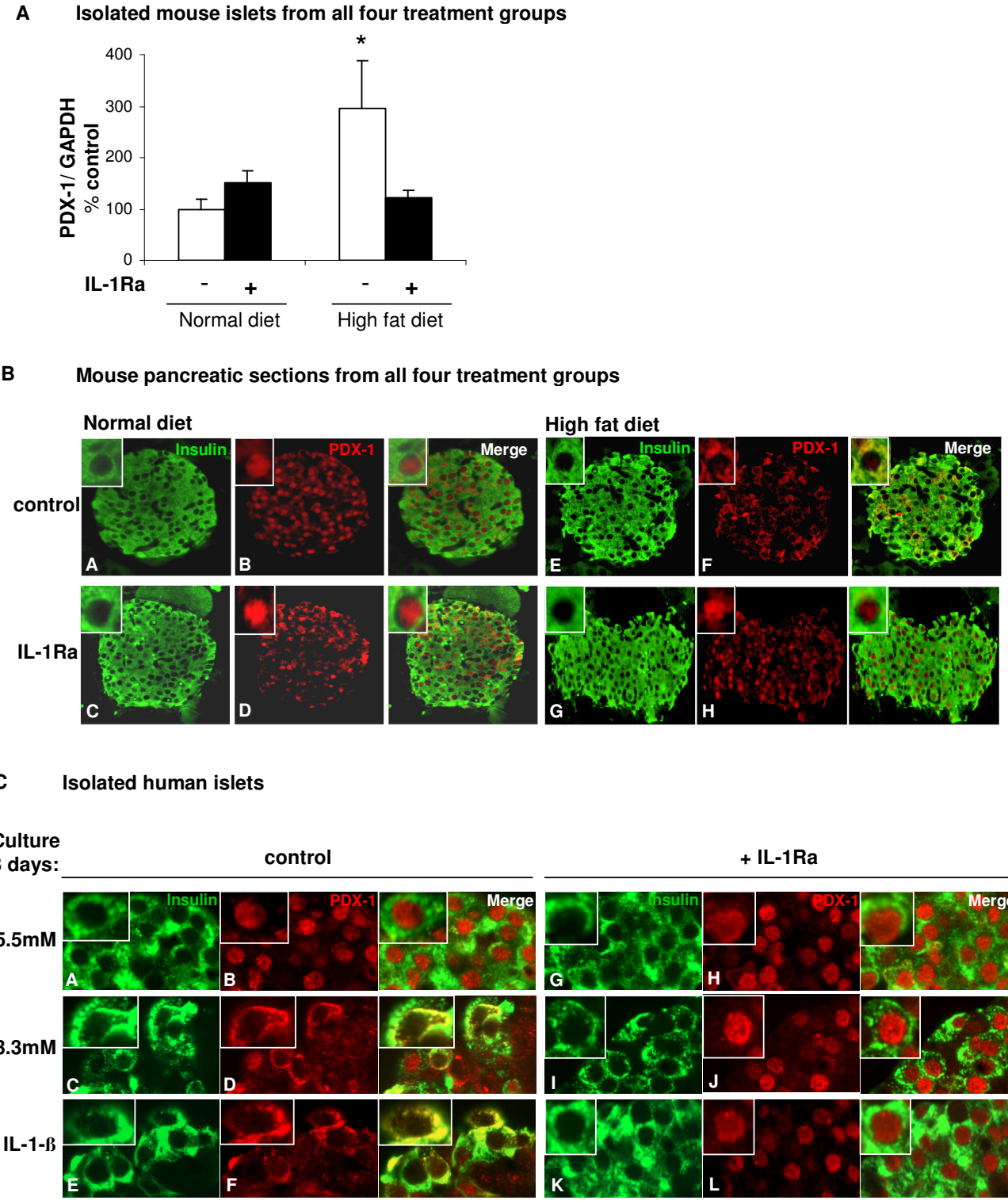

Figure 8: IL-1Ra treatment prevents PDX-1 translocation to the cytoplasm in mouse and human islets
(A) Quantitative RT-PCR analysis of PDX-1 expression relative to control ND conditions was performed in mouse islets isolated from the four treatment groups. PDX-1 levels were normalized to GAPDH and tubulin with the same result. Islets were isolated from 4 mice per treatment. Data are shown as mean ± SE, *p<0.05 HFD vs. ND vehicle. **(B)** Double immunostaining for insulin (green, panel A, C, E and G) and PDX-1 (red, panel B, D, F and H) in mouse pancreatic tissue sections from all four treatment groups, staining was performed from four different pancreases per treatment group (magnification x250, insert x2000). **(C)** Human isolated islets were treated for 72 hours with 5.5 mM glucose (control), 33.3 mM glucose or 2 ng/ml IL-1β with or without 500 ng/ml recombinant human IL-1Ra. Fixed and paraffin embedded islets sections were double-stained for insulin (green, panel A, C, E, G, I, K) and PDX-1 (red, panel B, D, F, H, J, L) and analyzed under the confocal microscope (magnification x1000, insert x4000). Islets were isolated from three different donors.

IL-1Ra treatment prevents PDX-1 translocation to the cytoplasm in mouse and human islets.

The molecular mechanism behind insulin gene regulation was studied by performing RT-PCR for the insulin transcription factor pancreatic duodenal homeobox-1 (PDX-1). While insulin mRNA in islets from high fat diet animals was decreased to 27% of control islets (Fig. 5E), PDX-1 levels were increased (Fig. 8A). IL-1Ra inhibited such increase. Since it has been reported that PDX-1 activity is primarily regulated by its sub-cellular localization (48; 49), we stained pancreatic tissue sections for PDX-1 and insulin. Sections from both normal diet groups showed PDX-1 immunoreactivity predominantly in the nucleus of β-cells (Fig. 8B). In contrast, islets under the high fat diet expressed PDX-1 exclusively in the cytoplasm. IL-1Ra treatment inhibited this translocation to the cytoplasm and the islets showed more prominent staining in the nucleus. To investigate whether our results on the mouse HFD model can be translated into mechanisms in human cells, we analyzed the effect of IL-1Ra on PDX-1 localization in human islets (Fig. 8C). Isolated human islets were treated with increasing glucose concentrations (5.5, 11.1 and 33.3 mM glucose) or 5.5 mM glucose plus 2 ng/ml IL-1β for 72 hours. As reported in many publications and in our previous studies (46; 50) (data not shown), under conditions of elevated glucose levels or IL-1β treatment β-cells failed to adequately increase insulin secretion in response to a glucose challenge. As shown in Fig. 8C, upper panel, in control conditions at 5.5 mM glucose, PDX-1 is localized in the nucleus in the insulin positive β-cells. Co-exposure of the islets to IL-1Ra did not change PDX-1 localization. Increasing glucose concentrations to 11.1 (data not shown) or 33.3 mM (Fig. 8C, middle panel) or addition of IL-1β (Fig. 8C, lower panel) shifted the PDX-1 signal almost exclusively to the cytoplasm, resulting in co-localization of PDX-1 with insulin (yellow merged color), whereas co-incubation with IL-1Ra protected from such PDX-1 shift; PDX-1 remained in the nucleus in the IL-1Ra treated islets (Fig. 8C right panel).

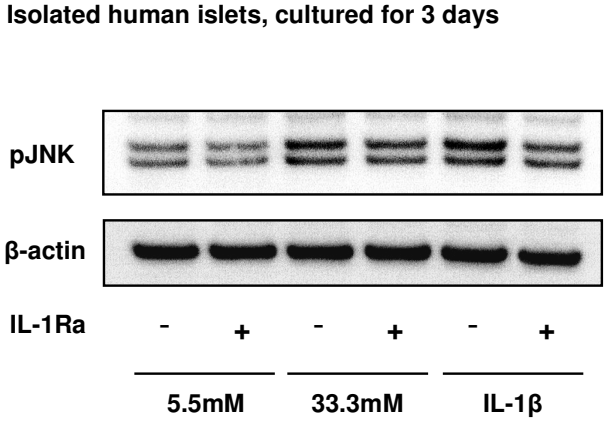

Figure 9: IL-1Ra prevents glucose- and IL-1β induced prolonged JNK-activation in human islets. Isolated human islets were cultured in suspension for 72 hours in 5.5mM glucose (control), 33.3mM glucose or 2ng/ml IL-1β with or without the addition of 500ng/ml recombinant human IL-1Ra. Immunoblotting of phosporylated JNK and β-actin (loading control). The antibodies were blotted on the same membrane. One representative blot of three experiments from three donors is shown.

IL-1Ra prevents prolonged glucose- and IL-1β-induced JNK-activation.

It has been shown that PDX-1 nuclear export is induced by oxidative stress through a signaling pathway that involves c-Jun N-terminal kinase (JNK) and forkhead transcription factor (Foxo) activity (1; 51; 52). Therefore, we investigated whether these cellular components also mediate glucose- and IL-1β-induced effects on PDX-1 localization by first evaluating whether IL-1Ra regulates JNK-activation. In isolated human islets, phosphorylated JNK-levels were increased by a 3-day culture in 33.3mM glucose or in the present of IL-1β (Fig. 9, upper blot) compared to control cultured at 5.5mM glucose. In both cases, this increase was inhibited when IL-1Ra was added to the culture medium. We confirmed these findings in isolated mouse islets. JNK remains in its activated state over a 3-day period in the presence of elevated glucose concentrations (Fig. 10, left panel, upper blot). In islets from mice that endogenously overexpress IL-1Ra (IL-1Ra-OE), glucose induces transient JNK phosphorylation after 1 day of culture, whereas after 3 days, JNK activation decreases (Fig. 10, right panel, upper blot).

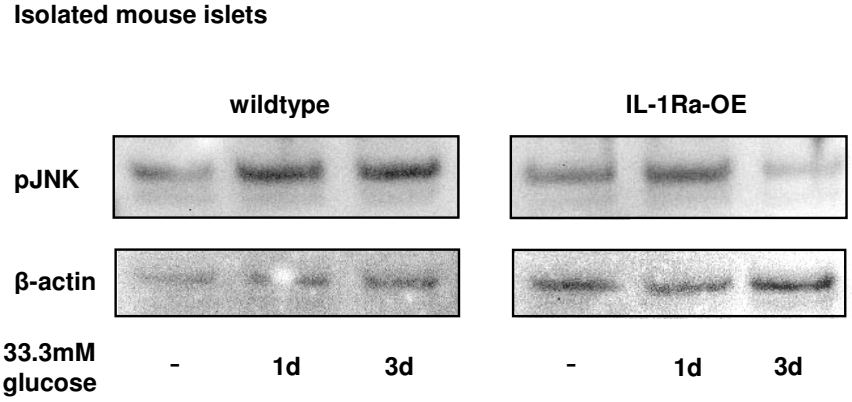

Figure 10: IL-1Ra-overexpression prevents glucose-induced prolonged JNK-activation in isolated mouse islets. Isolated islets from mice overexpressing IL-1Ra (IL-1Ra-OE) and wildtype littermates were treated for 1 or 3 days with 11.1mM glucose (control) or 33.3mM glucose. Immunoblotting for phosphorylated JNK and β-actin (loading control). The antibodies were blotted on the same membrane. One representative experiment of three is shown.

JNK-inhibition prevents glucose- and IL-1β-induced nuclear export of PDX-1.

Since we postulate that glucose-induced JNK-activation leads to PDX-1 export, JNK-inhibition would prevent shuttling despite the presence of elevated glucose or IL-1β. To test this hypothesis, we treated human islets with 33.3 mM glucose or with 2 ng/ml IL-1β, with or without the addition of JNKi, a small peptide that inhibits JNK-activity. We again observed glucose- and cytokine-mediated PDX-1 translocation (Fig. 11, panels D,F). We not only found cells that co-express insulin and PDX-1 in the cytoplasm, there are also cells that show cytoplasmic PDX-1 with almost no detectable insulin anymore (Fig. 11, arrow panel C,D and merge). In addition, some cells display PDX-1 in the nuclear periphery, which has been

previously observed in cell lines kept at low glucose concentrations ((53), Fig. 11, arrow panel F). JNK-inhibition prevented glucose- and IL-1β-induced PDX-1 translocation and kept the transcription factor in the nucleus in most cells, indicating that the JNK-pathway is involved in regulating PDX-1 localization.

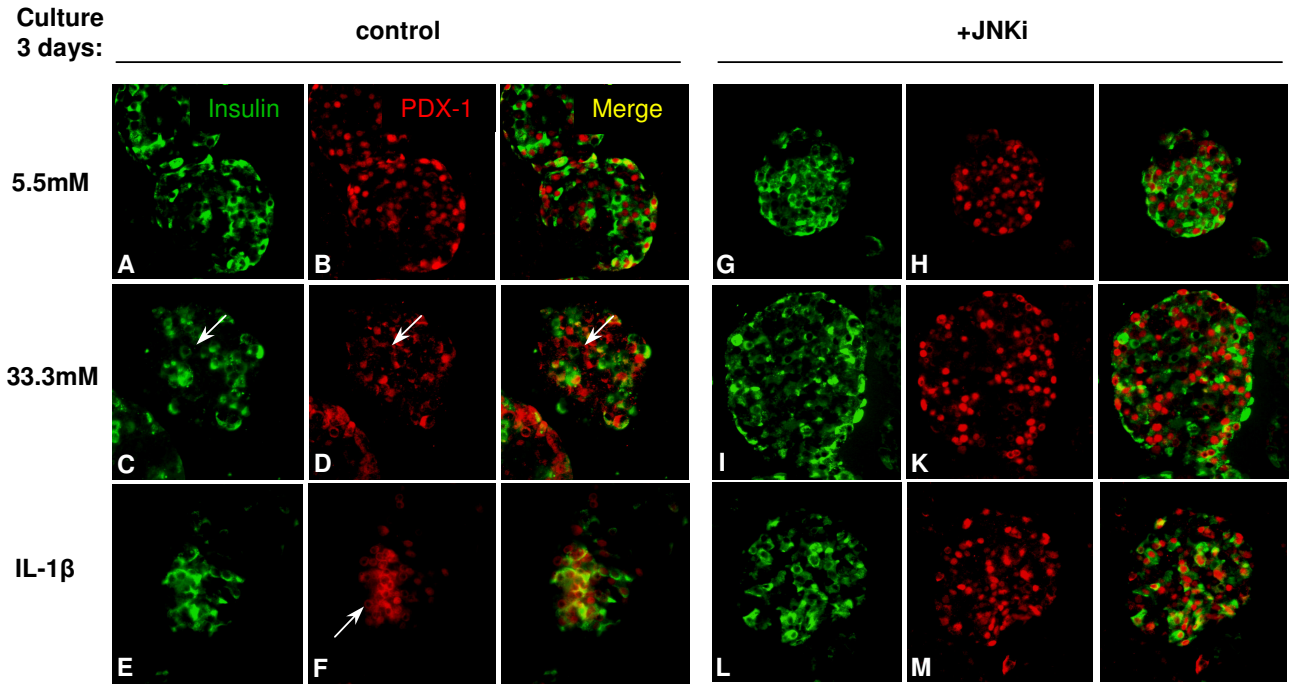

Figure 11: JNK-inhibition prevents PDX-1 translocation to the cytoplasm in human islets.
Human isolated islets were treated for 72 hours with 5.5 mM glucose (control), 33.3 mM glucose or 2 ng/ml IL-1β with or without 500 ng/ml recombinant human IL-1Ra. Fixed and paraffin embedded islets sections were double-stained for insulin (green, panel A, C, E, G, I, K) and PDX-1 (red, panel B, D, F, H, J, L) and analyzed under the confocal microscope. (magnification x1000, insert x4000). Islets were isolated from three different donors and experiments were performed in three independent experiments.

4.4.3 IL-1Ra Delays the Onset of Diabetes in the db/db-Mouse

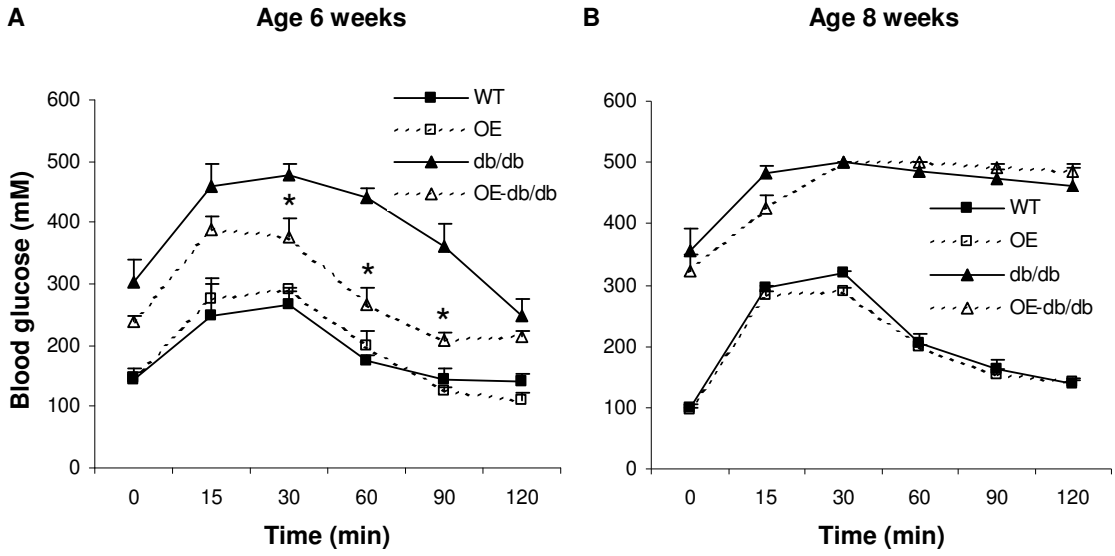

Figure 12: IL-1Ra overexpression delays the onset of diabetes in db/db-mice.
Blood glucose levels during IPGTT experiments in mice of 6 **(A)** and 8 **(B)** weeks of age. Glucose was i.p. injected at a concentration of 1 g/kg body weight. Data show mean ± SE. *p<0.05 db/db compared to OE-db/db. Data were collected from 8 animals per group, respectively.

IL-1Ra overexpression delays the onset of diabetes in db/db-mice.

To investigate the ability of IL-1Ra to protect from diabetes in another mouse model, we examined the effects of IL-1Ra in $Lepr^{db/db}$ mice (db/db). Homozygous mice on the C57BLKS/J background with this depletion in the leptin receptor become obese, hyperglycemic and hyperinsulinemic within the first month of age. It has been shown in these mice that IL-1β-mediated innate immunity is augmented which results from a diabetes-associated loss of IL-1β counteregulation (54). To test the hypothesis that IL-1Ra would prevent diabetes progression in this model of type 2 diabetes, we injected db/db-mice daily with IL-1Ra or with vehicle from 4 weeks of age on. Db/db mice which received IL-1Ra showed improved glucose tolerance during IPGTT experiments after 2 weeks of treatment as compared to their vehicle-treated littermates ($p<0.05$ at time points 30 and 60 minutes, data not shown). From 3 weeks of treatment on, we did not observe any differences in glucose levels among the two groups anymore. Considering the short half life of IL-1Ra (6 to 8 hours) and the 10- to 100-fold excess that is needed to block IL-1β-mediated effects (24), we tested whether constitutiv endogenous overexpression of IL-1Ra would improve the outcome of elevated IL-1Ra levels in db/db-mice. By crossbreeding mice which endogenously

overexpress IL-1Ra (OE) to C57BLKS-Lepr^{db} we obtained four groups of mice: IL-1Ra-OE-Lepr$^{db/db}$ (OE-db/db) and IL-1Ra-OE-Lepr$^{+/+}$ (OE) as well as their littermates without IL-1Ra-overexpression, Lepr$^{db/db}$ (db/db) and Lepr$^{+/+}$ (WT). As shown in Figure 12, IL-1Ra-overexpression delayed the onset of diabetes in db/db mice as their glucose tolerance is improved up to 6 weeks of age compared to db/db mice without IL-1Ra overexpression (Fig. 12A). As observed in mice injected with IL-1Ra, this protection is a transient state as at 8 weeks of age, there is no difference in glucose tolerance despite the constitutiv overexpression of IL-1Ra (Fig. 12B).

4.5 DISCUSSION

Elevated glucose concentrations impair β-cell function and induce apoptosis and thus are implicated in accelerating the progression of diabetes. Strategies to block these deleterious effects on the β-cell are needed for a successful diabetes therapy. Interleukin-1 Receptor Antagonist has been shown to inhibit the deleterious effects of glucose on β-cells by blocking pro-inflammatory IL-1β signaling *in vitro* (17). Here we show that IL-1Ra improved glucose tolerance, insulin secretion, and insulin sensitivity in C57BL/6J mice fed a high fat/ high sucrose diet (Surwit), serving as an animal model of T2DM. Twelve weeks of high fat feeding resulted in hyperglycemia accompanied by impaired glucose-stimulated insulin secretion. Interestingly, the changes induced by the high fat feeding strongly correlated with the individual weight gain of the mice. For instance, a mouse with a 1.15-fold higher body weight in our study (46 g vs. 40 g) also had 1.12-fold higher glucose levels at all time-points during the IPGTT ($p<0.05$, data not shown). This strong association of glucose tolerance and body weight may explain the relatively large variation we observed throughout the study, which has been reported before in a similar diet-induced obesity model (55).

Long-term high fat diet in C57BL/6J mice is associated with an adaptive increase in β-cell number but an early functional abnormality (56). In the present study, β-cell mass increased over 2-fold during the twelve weeks of high fat/ high sucrose diet. Despite this increase in β-cell mass, β-cell turnover was already impaired at that time point. We assume that the combination of high fat and high sucrose in the diet results relatively early in an impairment of β-cell turnover together with loss of function. In line with this, massive reduction in β-cell replication after long-term high fat/ high carbohydrate diet (decreased to one third of the control group) has been observed in mice before a significant decrease in β-cell mass (57). Consistent with our present study, ~0.3% proliferating β-cell were observed in the control mice (57) and low numbers as 0.025% proliferating β-cells were reported from human pancreatic sections from autopsy (6). Proliferation in adult β-cells is a rare event, but small changes in β-cell proliferation or apoptosis over the years can result in marked changes in β-cell mass and diabetes progression (6; 57).

Previous studies observed that glucose induces increased proliferation in short-term culture, but decreases proliferation after long-term exposure of isolated islets (58). We hypothesize that in our model, a transient rise in diet-induced proliferation occurred earlier in the study, mediated by mildly elevated glucose levels, proceeding to decreased proliferation measured at 12 weeks, when severe hyperglycemia was present. IL-1Ra-treated mice were protected from this HFD-induced decrease in proliferation. Under the normal diet, IL-1Ra-injections resulted in increased β-cell proliferation after 12 weeks, consistent with *in vitro* data on isolated human islets (46). We measured only very few apoptotic β-cells *in vivo,* and could

not quantify significant changes among the four treatment groups. It is noteworthy that isolated islets derived from the HFD-group displayed a 3-fold induction of β-cell apoptosis compared to islets from the ND-group. This suggests that high fat feeding renders β-cells more susceptible to apoptosis induced by isolation procedures and/ or culture. This increased sensitivity was not observed in islets from IL-1Ra-treated HFD-mice.

The effect of IL-1Ra on improving glucose tolerance is probably a result of both improved function of the β-cell and insulin action. Especially, the effects of IL-1Ra serum lipids, adipokines and cytokines would in turn improve β-cell function as well as insulin sensitivity. Free fatty acids have been shown to directly activate pro-inflammatory signaling through TLR4 in adipose cells and macrophages (59). Also, free fatty acids (40) and triglycerides (60) induce β-cell dysfunction and apoptosis.

Several adipocytokines, e.g. leptin, resistin and adiponectin play a central role in the regulation of insulin resistance, as well as of inflammation, immunity and β-cell function (47; 61). In line with this we show pro-inflammatory leptin and resistin induced by the HFD, whereas adiponectin, which is known to prevent inflammation (61), was decreased by the HFD. All of these adipokines were normalized by IL-1Ra. Interestingly, adiponectin can directly inhibit IL-6 and TNF-α production and induce production of IL-1Ra by human monocytes, macrophages and dendritic cells (61). To analyze systemic levels of inflammatory cytokines in the mice, we measured IL-1 and IL-6 serum concentrations. Unfortunately, no ultra-sensitive assays for such measurements are available and therefore most of the values were under the detection levels of the assays. We detected positive IL-1 and IL-6 readings in some but not all samples from the HFD control mice. However, data analysis and statistics were not possible. On the other hand, serum IL-1Ra levels were unchanged by the HFD after 12 weeks. Therefore, we hypothesize that the ratio of IL-1/IL-1Ra plays an important role in maintaining glucose homeostasis.

Amounts of secreted leptin and free fatty acids are dependent on fat mass (62), but also on the direct effect of IL-1β on the adipocytes (63). The fact that we do observe changes in leptin and FFA secretion in IL-1Ra-treated HFD-mice without changes in fat mass and adipocyte size supports an IL-1 dependent effect. TNF-α and IL-1β expression in epididymal fat pads could trigger HFD-induced insulin resistance (64) and also negatively act on the β-cell (65; 66). Here we show mRNA levels of the inflammatory cytokines IL-1β and TNF-α, the macrophage marker F4/80 and the pro-inflammatory macrophage marker CD11c are increased by the HFD in wildtype mice but reduced by IL-1Ra overexpression. A HFD-induced pro-inflammatory state of adipocytes may be the reason for the increased leptin, FFA, cholesterol and triglyceride production in the HFD control mice. Even though IL-1Ra mRNA in adipocytes was 7.4-fold increased by the HFD, this could likely not counteract the 2.7-fold HFD-induced increase of IL-1β, since an excess of 10- to 1000-fold of IL-1Ra is

needed to inhibit IL-1β effects, dependent on the exposure time (17; 24). In contrast, in IL-1Ra-OE mice of both diet groups, IL-1β mRNA levels were significantly lowered, whereas IL-1Ra mRNA was increased. Our study provides more evidence for an existing link between inflammation, β-cell function and survival and insulin resistance. Undoubtedly, the effect of IL-1Ra on adipocyte-derived factors participated in the protective role of IL-1Ra on the level of the β-cell.

Increased IL-1Ra serum levels correlate with obesity and insulin resistance in humans (67), suggesting that IL-1Ra induces rather than protects from insulin resistance. Moreover, a negative correlation of elevated IL-1Ra and whole body glucose uptake has been shown in offspring of T2DM subjects. In addition, injecting rats with IL-1Ra for 5 consecutive days leads to decreased whole body glucose disposal due to a selective decrease in glucose uptake in the muscle (68). In our study, we have not assessed the short-term effects of IL-1Ra administration in mice. We had no indication that 12-week administration of IL-1Ra negatively influences insulin sensitivity. IL-1Ra treated ND mice showed similar insulin levels and insulin tolerance as the vehicle-treated controls. Moreover, under the high fat diet, IL-1Ra-mice had lower insulin levels than the control mice and improved insulin sensitivity. Possibly, the increased IL-1Ra serum levels in obese patients could be secondary in response to insulin resistance.

Investigating the underlying mechanisms of IL-1Ra-mediated improved glucose-stimulated insulin secretion in islets from the HFD-group, we found that IL-1Ra counteracted high fat diet-induced diminished insulin mRNA levels. Unexpectedly, PDX-1 mRNA levels were oppositely regulated to insulin levels, with increased PDX-1 levels induced by high fat feeding which were normalized in the IL-1Ra-treated HFD-group. The pancreatic duodenal homeobox PDX-1 is an important islet transcription factor, which controls the physiological expression of the insulin gene (69) and mediates the effects of glucose on insulin gene transcription (70; 71) as well as β-cell survival (72). Our results are in contrast to previous data showing PDX-1 downregulation in response to hyperglycemia in β-cells in culture (73) as well as in type 2 diabetic animal models, e.g. in ZDF rats (74), in psammomys obesus (75) and in partially pancreatectomized rats (76). However, studies with isolated islets from patients with T2DM have shown a similar opposite regulation, with reduced insulin mRNA in parallel to increased PDX-1 mRNA levels, compared to islets isolated from non-diabetic controls (77). Possibly, β-cell functional compensation can occur through increased PDX-1 mRNA. On the other hand, posttranslational changes, which define PDX-1 localization, rather than mRNA expression levels, may play a more important role under diabetic conditions (78). Upon acute exposure of β-cells to glucose, PDX-1 translocates from the cytoplasm/nuclear periphery to the nucleoplasm, leading to insulin gene transcription (53). In contrast, oxidative stress induces PDX-1 shuttling from the nucleus to the cytosol, and thus causes severe

reduction of PDX-1 activity (52). In our study, we detected PDX-1 predominantly expressed in the cytosol of the HFD-group. In both control groups (ND, ND+IL-1Ra) as well as in the IL-1Ra-treated HFD-group, PDX-1 was mostly localized in the nucleus. Since IL-1Ra protected from the pro-diabetic effect of the high fat diet, we propose that this is partly the result of maintaining PDX-1 functionally in the nucleus.

We show that, besides oxidative stress (52) and the combination of palmitic acid and elevated glucose (79), chronic hyperglycemia as well as the cytokine IL-1β induce PDX-1 nuclear export. In both conditions, IL-1Ra preserved nuclear PDX-1, in parallel to previously observed protection from β-cell failure (17). In this study, we identify JNK as cellular component involved in glucose- and IL-1β-regulated PDX-1 localization. In wildtype islets, JNK is phosphorylated after short-term (30min (data not shown) and 1 day) as well long-term (3 days) incubations with elevated glucose. Therefore, it seems that acute JNK-activation is beneficial for β-cell function, whereas chronic activation correlates with glucotoxicity. However, IL-1Ra protected islets from prolonged JNK-activation.

JNK-activity has previously been linked to PDX-1 shuttling under conditions of oxidative stress (52), together with Foxo1 as keyplayer (1). Foxo1 cellular localization determines PDX-1 localization, which is opposite to each other. Foxo1 itself is regulated by JNK and AKT-activity, JNK induces Foxo1 nuclear import, which leads to PDX-1 export, whereas AKT-mediated Foxo1 phosporylation results in Foxo1 cytoplasmic and PDX-1 nuclear localization. Our findings suggest the JNK-PDX-1 pathway as critical signaling network which transduces short-term as well as long-term glucose stimulation and therefore might partly mediate the dual effect of glucose on β-cells function and survival (Fig. 13).

The fact that IL-1Ra potentially prevented hyperglycemia and improved β-cell function is in favor for the critical role of IL-1β signaling in the β-cell not only in type 1 but also type 2 diabetic environments. Our data provide new insights into mechanisms of the protective effect of IL-1Ra on β-cell function and turnover and support IL-1Ra as a potential therapy of diabetes.

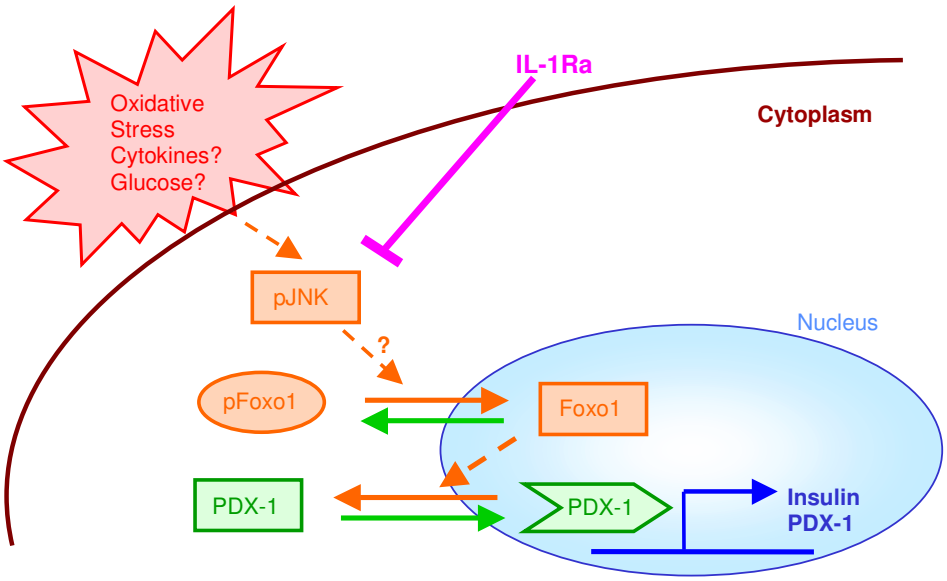

Figure 13: Model of IL-1Ra-regulated PDX-1 shuttling.
Extracellular signals lead to JNK phosphorylation and activation. As a consequence, pFoxo gets dephosphorylated and translocates into the nucleus. This results in export of PDX-1 from the nucleus to the cytoplasm. Foxo and PDX-1 are hypothesized to always be localized opposite to eachother, by a mechanism that might include direct protein-protein interactions (1). PDX-1 is a transcription factor that activates expression of genes such as insulin, glucokinase and glucosetransporter 2. It has also been shown to regulate its own expression.

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5. INHIBITION OF ERK1/2-ACTIVATION IS RESPONSIBLE FOR GLUCOSE - DESENSITIZATION OF HUMAN PANCREATIC ISLETS

A manuscript of this study will be submitted for publication.

5.1 SUMMARY

In type 2 diabetes, chronic overstimulation due to hyperglycemia is suggested to be detrimental to pancreatic β-cell-function and survival, leading to the concepts of β-cell desensitization and glucotoxicity. It has been shown that the mitogen-activated protein-kinases ERK1/2 are activated by increased concentrations of glucose and that their activation is crucial for β-cell function via activation of the insulin promoter. However, it is not known if chronic hyperglycemia affects ERK1/2 signaling in the β-cell. The aim of the present study was to determine if glucose-induced β-cell desensitization is associated with loss of ERK1/2 activity in order to find a target to protect from the deleterious effects of glucose on β-cell function.

Human islets were maintained in suspension culture for 3 days at 5.5, 11.1 or 33.3 mM glucose with or without the addition of Diazoxide, Somatostatin or PD98059. After the incubation period, the islets were challenged with an additional short-term (30 minutes) glucose-incubation at 5.5 mM or 33.3 mM, respectively, to address their glucose responsiveness. ERK1/2-activation was measured using antibodies that selectively recognize the phosphorylated forms of the kinases. In parallel, insulin secretion into the culture medium was evaluated to assess β-cell function.

Human islets, maintained in culture at 5.5 mM glucose, showed a 3-fold glucose-stimulated insulin secretion as well as a 5-fold stimulation of ERK1/2-phosphorylation. In contrast, islets cultured for three days at 11.1 or 33.3 mM glucose showed a competely abolished stimulatory index, together with a decrease in ERK-activation in response to a glucose challenge compared to control islets cultured at 5.5 mM glucose.

We tested the hypothesis that inhibiting overstimulation would induce β-cell rest and restore its function. Therefore, we co-incubated the islets with diazoxide or somatostatin during the three-day culture period, which almost completely inhibited insulin secretion. Subsequently, short-term glucose challenge for 30 minutes induced insulin secretion and western blot analysis showed that glucose was able to upregulate ERK1/2-phosphorylation.

Finally, and in support of our results, we demonstrated that co-incubation of the islets with the ERK1/2 inhibitor PD98059 also prevented decreased ERK1/2-activation as well as

impaired stimulated insulin release after the additional glucose challenge, indicating that PD98059 induces β-cell rest similar to Diazoxide.

We conclude that chronic exposure of human islets to elevated glucose levels desensitizes the β-cell to further glucose stimulation and inhibits ERK1/2-activation. Inhibition of β-cell overstimulation, by blocking either insulin secretion or ERK1/2 phosphorylation during the chronic culture, restores glucose induced ERK1/2 activation together with β-cell function. Our data provide a new mechanism of glucose desensitization.

5.2 INTRODUCTION

Glucose is a key modulator of β-cell function and survival that regulates an array of cellular signaling networks. In the short-term, glucose metabolism in the β-cell itself generates a rise in the ATP to ADP ratio that results in closure of ATP-dependent potassium channels, followed by membrane depolarization and opening of voltage-operated Ca^{2+} channels. The consequent Ca^{2+}-influx triggers insulin release by exocytosis of insulin-containing secretory granules. Despite this well-recognized mechanism, calcium entry has been reported to also activate various kinases in the β-cells, including the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (1; 2). In isolated islets, increasing glucose concentration markedly increases the levels of the phosphorylated forms of ERK1/2 (3). ERK1/2 are ubiquitous enzymes with many substrates that are involved in a variety of cellular events such as proliferation, differentiation and survival (4). Besides the calcium-dependent activation of ERK1/2 by glucose, insulin and growth factors regulate ERK1/2 in β-cells in a largely calcium-independent manner (5). It has been shown that insulin transcription is dependent on ERK1/2 as blockade of ERK1/2-activity inhibited basal as well as glucose-stimulated insulin promoter activity (6). In addition to an involvement of ERK1/2 on glucose-stimulated insulin transcription, the kinases have also been implicated in the process of insulin secretion. Longuet et al. (7) showed that glucose-induced activated ERK1/2 remains to a large part in the cytoplasm. Therefore, phosphorylated ERK1/2 seems to exert other functions beside gene regulation in the nucleus. Indeed, blocking activation of ERK1/2 resulted in partial inhibition of glucose-induced insulin release. Furthermore, cytoplasmic pERK1/2 interacts with synapsin I. Synapsin I phosphorylation by protein-kinase A (PKA) and calmodulin kinase II (CAMKII) has been linked to mediate glucose-induced insulin release (8; 9). This interaction only occurred between synapsin I and the phosphorylated, active form of ERK1/2 and resulted in serine phosphorylation of synapsin I, implicating a participation of ERK1/2 in acute glucose-stimulated insulin secretion.

In contrast to short-term effects, chronically elevated glucose can lead to β-cell desensitization. Desensitization is a temporary physiological state of cellular refractoriness to glucose stimulation that, in contrast to glucotoxicity, can be reversed after restoration of normal glucose concentrations. Parts of the toxic effects of hyperglycemia are thought to be due to chronically elevated intracellular Ca^{2+}-concentrations. Inducing 'β-cell rest' by potassium channel openers or calcium channel antagonist might therefore preserve β-cell function in the presence of prolonged high glucose. Already in 1976, there was evidence that K_{ATP} channel openers may have protective effects on β-cells since diabetic patients which received the K_{ATP} channel opener diazoxide for 7 days showed imroved insulin secretion (10). These findings were confirmed in more recent studies with type 1 (11) and type 2 (12) diabetic patients. Moreover, diazoxide also protected β-cells *in vitro* from the deleterious effects of high glucose as well as IL-1β (13). Addition of diazoxide to the chronic culture medium prevented glucose- and IL-1β-induced impaired function and apoptosis. Interestingly, blocking ERK1/2-activation by PD098059 also inhibited diminished function and increased β-cell death. However, it is not known whether glucose desensitization after chronic hyperglycemia also affects the responsiveness of ERK1/2 to a short-term glucose challenge and whether restoration of β-cell function after normalization of glucose concentrations also involves a recovered ability to phosphorylate and activate ERK1/2 in response to glucose. Therefore, we investigated basal and glucose-stimulated pERK1/2 levels as well as insulin secretion into the supernatant of islets after chronic incubation in control and elevated glucose conditions with or without the addition of agents that inhibit insulin secretion or ERK1/2-activity.

5.3 EXPERIMENTAL PROCEDURES

Islet isolation and culture. Human islets were isolated at the University of Illinois at Chicago as previously described (14). The islet purity was >95% as judged by dithizone staining. When this degree of purity was not primarly achieved by routine isolation, islets were handpicked. In all experiments, islets were pre-cultured for 24 hours after receiving the islets and before starting experiments. Groups of 100 human islets were cultured in suspension culture dishes in CMRL-1066 medium containing 5.5 mM glucose supplemented with 100 U/ml penicillin, 100µg/ml streptomycin and 10 % FCS (Invitrogen Ltd., Carlsbad, CA, USA), hereafter referred to as culture medium. After the preincubation time, the medium was changed to culture medium containing 5.5, 11.1 or 33.3 mM glucose with or without the addition of 100 µM Diazoxide (Sigma-Aldrich, St. Louis, MO, USA), 1 µM PD098059 (Calbiochem, San Diego, CA, USA) or 50nM Somatostatin (Bachem, Torrance, CA, USA) for 72 hours. For recovery experiments, medium was again changed to culture medium containing 5.5 mM glucose for an additional two days.

Acute insulin release and ERK1/2 phosphorylation. For acute insulin release in response to glucose, islets were washed in Kreb's Ringer bicarbonate buffer (KRB) containing 5.5 mM glucose and 0.5% BSA. KRB was then replaced by KRB 5.5 mM glucose (basal) or by KRB 33.3 mM glucose (stimulated) for 30 minutes with or without the addition of 100 nM somatostatin (to investigate the influence of secreted insulin on ERK1/2 activation). Acute supernatants were collected and stored at –80°C until analysis for insulin concentration by human insulin ELISA (Dako, Carpinteria, CA, USA). Islet pellets were harvested for Luminex assays, Western blotting or RT-PCR quantification.

Detection of Phosphoproteins. After the respective chronic and acute incubation periods, phosphoprotein levels in the islets were measured using Bio-Plex Luminex technology (Bio-Rad Laboratories, Hercules, CA, USA), accoring to the manufacturer's instructions. The premixed multiplex assays were designed to detect levels of phosphorylated GSK, ATF2, JNK, p38, ERK1/2, STAT3, IκB, AKT.

Western blot analysis. At the end of the chronic and acute incubation periods, islets were washed in ice-cold PBS and lysed for 40 minutes on ice in 40 µl lysis buffer containing 20 mM Tris acetate, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate. Prior to use, the lysis buffer was supplemented with Protease- and Phosphatase-inhibitors (Pierce, Rockford, IL, USA). Equal amounts of protein of each treatment group were run on NuPAGE 4-12% Bis-Tris gels.

Proteins were electrically transferred to PVDF membranes. Blocking of non-specific protein binding was achieved by incubating the filter membrane in blocking buffer (1xTBS, 0.1% Tween-20, 5% BSA). After washing in 1xTBS, 0.1% Tween-20 (TBS-T), membranes were incubated with rabbit anti-phospho p44/42 MAP-kinase (Thr202/Tyr204) antibody overnight at 4°C followed by incubation with horseradish-peroxidase-linked anti-rabbit IgG.

RNA extraction and quantitative RT-PCR analysis. Total RNA of isolated islets was extracted after overnight culture as described previously (15). For quantitative analysis, we used the LightCycler Quantitative PCR System (Roche) with a commercial kit (LightCycler FastStart DNA Master plus SYBR Green I, Roche). Human primers used were 5'CTACCTAGTGTGCGGGGAAC3' and 5'GCTGGTAGAGGGAGCAGATG3' (Insulin) and compared to the house keeping gene 5'AGAGTCGCGCTGTAAGAAGC3' and 5'TGGTCTTGTCACTTGGCATC3' (α-Tubulin).

5.4 RESULTS

Chronically elevated glucose levels lead to loss of acute glucose-stimulated phosphorylation of ATF2 and ERK1/2.

To investigate which proteins are affected by chronically elevated glucose levels, we cultured isolated human islets at 5.5 mM (control) or 33.3 mM glucose for 3 days. To address their glucose responsiveness after this incubation time, they were exposed to an additional short-term glucose challenge for 30 minutes at basal (5.5 mM) or stimulated (33.3 mM) conditions, immediately followed by protein extraction. We then performed bead-based multiplex assays for several phosphoproteins. We found 3 proteins whose acute glucose-induced phosphorylation was diminished when islets were previously exposed to chronically high glucose: the transcription factor ATF2 (Fig.1A) and the kinases ERK1/2 (Fig.1B) and AKT (data not shown). Short-term glucose (30 minutes at 33.3 mM glucose) after 3-day culture under control conditions enhanced phosphorylation status of these proteins significantly compared to basal. There was no acute glucose-stimulated activation when islets were kept in elevated glucose concentrations of 33.3 mM for 72 hours.

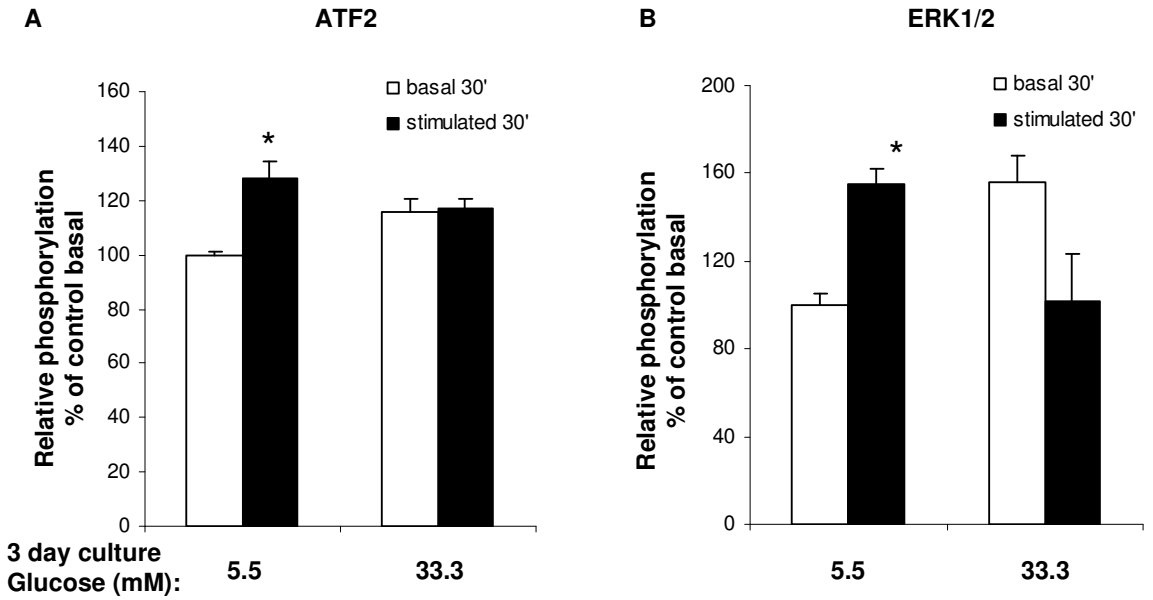

Figure 1: Chronic hyperglycemia diminishes acute glucose-stimulated phosphorylation.
Human islets were cultured in suspension dishes for 72 hours in 5.5 mM or 33.3 mM glucose, followed by an acute glucose incubation for 30 minutes at basal (5.5 mM) or stimulated (33.3 mM) conditions. Islet proteins were extracted and evaluated for phosphorylation levels of **(A)** ATF2 and **(B)** ERK1/2 using Luminex technology. Data show mean ± SE. *p<0.05 stimulated compared to basal phosphorylation. Analysis was performed in three experiments from three organ donors.

We confirmed these findings by Western blotting with a phospho-specific antibody that only recognizes ERK1/2-proteins that are phosphorylated at positions 202 (Thr) and 204 (Tyr). As seen in Fig. 2, following culture at 5.5 mM glucose, islets respond to a 30min glucose challenge with upregulating phospho-ERK1/2 levels (left panel), whereas chronic hyperglycemia (middle and right panel) abolishes acute ERK1/2-activation.

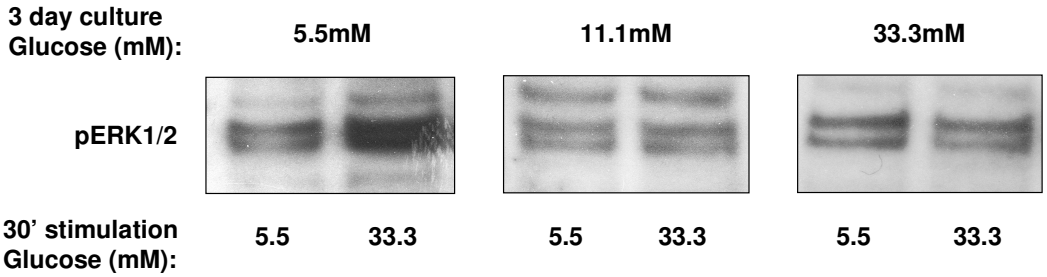

Figure 2: Chronically elevated glucose levels lead to loss of ERK1/2-activation after glucose challenge.
Western blot analysis of pERK1/2. Human islets were cultured in suspensiond dishes for 72 hours at 5.5, 11.1 or 33.3 mM glucose, followed by acute 30 minutes stimulation with 5.5 or 33.3 mM glucose in KRB. One representative blot out of six experiments from six organ donors is shown.

Inhibition of insulin secretion and ERK1/2-activity during chronic incubation restores acute ERK1/2-activation.

To test the hypothesis that inhibiting overstimulation would induce β-cell rest and protect function, we added diazoxide to the culture medium during the chronic period which inhibited insulin secretion. Subsequently, short-term high glucose was able to enhance ERK1/2-phosphorylation compared to basal (Fig.3 second panel). Co-incubation with PD098059 during the 3 days, an inhibitor of MEK1/2, also prevented decreased acute ERK1/2-activation, indicating that ERK1/2 inhibition induces β-cell rest comparable to Diazoxide (Fig.3, third panel).

Acute ERK1/2-activation does not depend on secreted insulin.

It has been shown that ERK1/2 can be activated by glucose and by insulin but through different pathways (reviewed in (5)). Hence we investigated whether pERK1/2-upregulation is a direct result of glucose-stimulation and or whether it depends on secreted insulin. In consequence, we added somatostatin to the islets during the chronic as well as during the acute period. In that, insulin secretion was prevented for the long-term culture and for the 30 minutes stimulation time. Inhibiting insulin secretion had no effect on ERK1/2-activation (Fig.3, lowest panel). In islets cultured at 5.5 mM glucose, there is a still strong acute glucose-induced pERK1/2 signal despite abolished insulin secretion. In addition, in cells

treated with 11.1 mM glucose, we observed restoration of glucose-stimulated ERK1/2-activity showing independece between ERK-phosphorylation and secreted insulin.

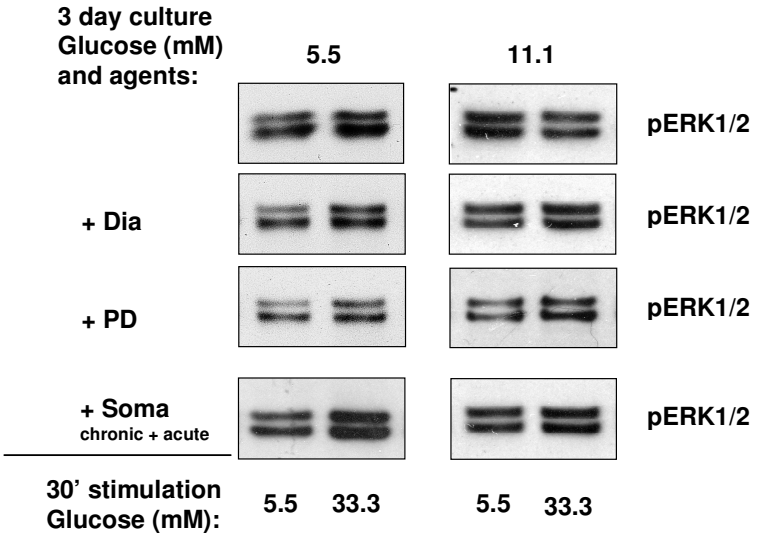

Figure 3: Prevention of hyperglycemia-induced loss of acute ERK1/2-phosphorylation.
Isolated human islets were cultured for 72 hours in suspension dishes at 5.5 or 11.1 mM glucose with or without the addition of 100 μM Diazoxide, 1 μM PD098059 or 50 nM somatostatin. Chronic incubation was followed by 30 minutes acute stimulation in KRB containing 5.5 mM or 33.3 mM glucose. For experiments investigating the influence of secreted insulin, somatostatin at a concentration of 100 nM was also added to the acute stimulation with KRB. Islet protein was extracted and equal amounts were analyzed by Western blotting for pERK1/2 levels. One representative experiment of four experiments from four organ donors is shown.

Desensitized islets cultured at 11.1mM glucose can regain acute glucose-stimulated ERK1/2 phosphorylation after recovery.

Since glucose desensitization is a temporary state that can be reversed upon restoration of normoglycemia, we postulated that in parallel to glucose sensing, islets would also regain the ability to respond to glucose by activating ERK1/2. To test this hypothesis, after chronic incubation at 5.5, 11.1 or 33.3 mM glucose for 3 days, islets were cultured for another 2 days at 5.5 mM glucose to allow them to recover from chronic hyperglycemia before acute glucose-stimulation. 2 days under normal glucose concentrations restored acute ERK1/2 phosphorylation in islets that were previously treated with 11.1 mM glucose for 3 days (Fig.4, middle panel). However, 2 days of recovery following chronic incubations at 33.3 mM was not able to reverse the adverse effects of glucose, as the islet cells did not upregulate active ERK1/2 in response to the glucose challenge (Fig.4, right panel).

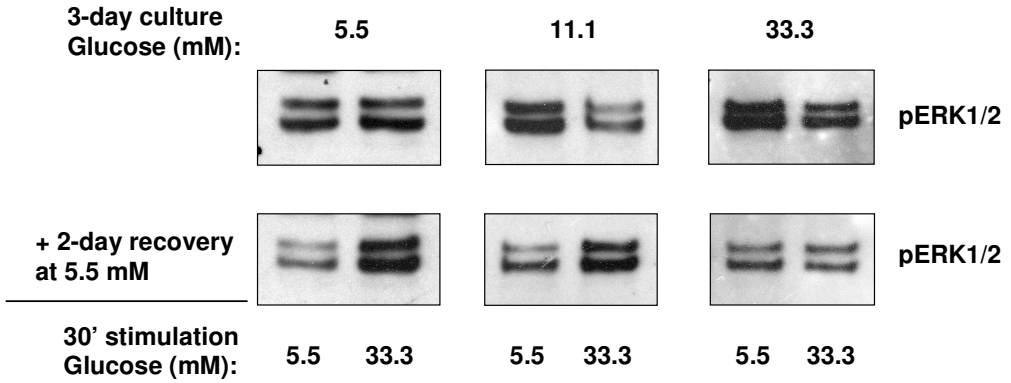

Figure 4: Recovery of glucose-desensitized islets.
Human islets were cultured in suspension dishes for 72 hours at 5.5, 11.1 or 33.3 mM glucose. Following this chronic incubation, islets were allowed to recover for an additional 2 days at 5.5 mM glucose before assessing acute glucose responsivness for 30 minutes at 5.5 or 33.3 mM glucose in KRB. Islet protein was extracted and analyzed by Western blotting for pERK1/2 levels. One representative blot from 3 experiments from 3 organ donors is shown.

Glucose-stimulated acute ERK1/2-activation correlates with insulin secretion.

We postulated that prevention of decreased glucose-stimulated ERK1/2 phosphorylation by inducing β-cell rest would in parallel restore insulin secretion. To test this hypothesis, we measured secreted insulin in the supernatants of the above-described experiments (Figures 2-4). As shown in Figure 5, human islets cultured in control conditions (5.5 mM glucose) increased their insulin secretion 2-3 fold in response to a glucose challenge. This increase was completely abolished when the islets were kept at 11.1 mM glucose for 72 hours. Addition of diazoxide or the ERK1/2-inhibitor PD098059 to the chronic culture medium containing 11.1 mM glucose, counteracted this effect as seen by a restored stimulation of insulin release induced by glucose which is consistent to restored ERK1/2 phosphorylation. As a control and as expected, adding somatostatin to the acute stimulation completely inhibited insulin secretion. Nevertheless, as shown by western blot (Fig.3), this does not affect glucose-induced ERK1/2-activation. In addition, recovery from 11.1 mM glucose not only resensitizes the islets for acute glucose-stimulated pERK upregulation (Fig.4) but also for stimulation of insulin secretion.

Acute effects of glucose do not result in increased mature insulin RNA levels.

Since it has been shown that acute glucose stimulates insulin transcription in a ERK1/2-dependent manner (3; 6), we investigated mature insulin RNA levels in our experimental settings. Total RNA was isolated from human islets incubated at 5.5 mM or 11.1 mM glucose for 3 days, followed by stimulation at 5.5 (basal) or 11.1 (stimulated) mM glucose for 1 hour. RT-PCR revealed that there is no detectable change in insulin mRNA concentrations in response to glucose stimulation in islets previously cultured at 5.5 or 11.1 mM glucose.

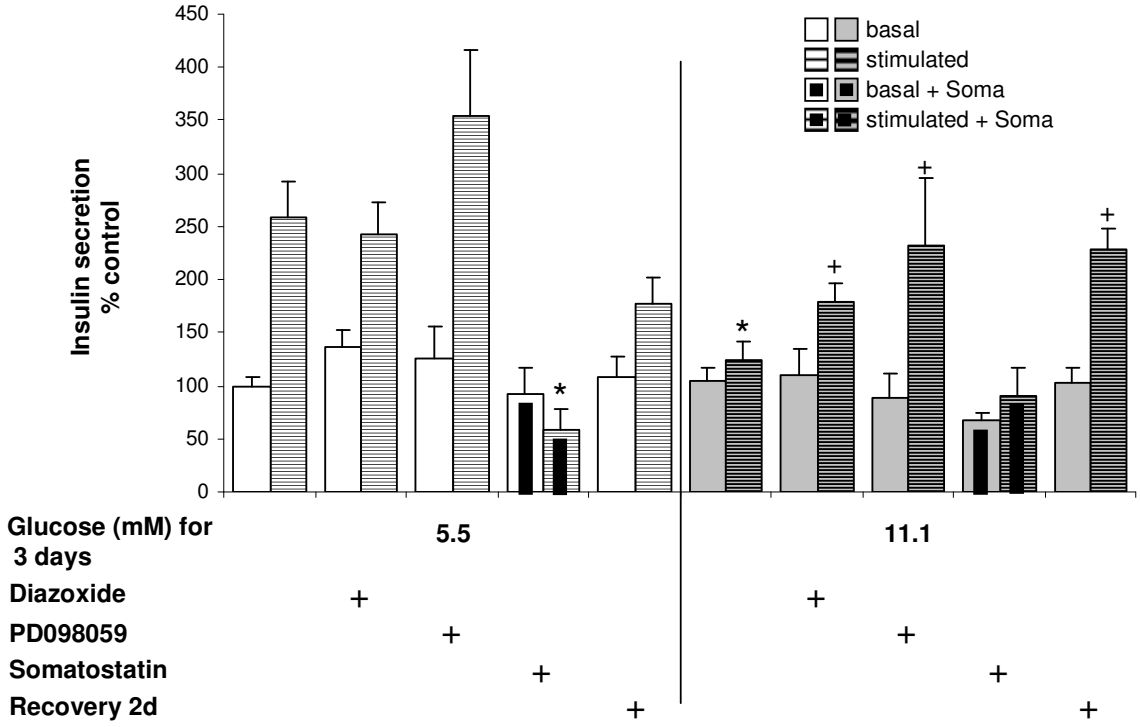

Figure 5: Prevention of hyperglycemia-induced impaired GSIS.
Human islets were cultured for 72 hours at 5.5 or 11.1 mM glucose with or without the addition of 100 μM Diazoxide, 1 μM PD098059 or 50 nM Somatostatin. This chronic incubation was followed by 30 minutes acute stimulation at 5.5 mM (basal) or 33.3 mM (stimulated) glucose with or without the addition of 100 nM somatostatin. Secreted insulin in the supernatant was measured using human insulin ELISA. Data show mean ± SE. *p<0.05 compared to control stimulated, $^{+}$p<0.05 compared to stimulated levels after 3-day glucose treatment.

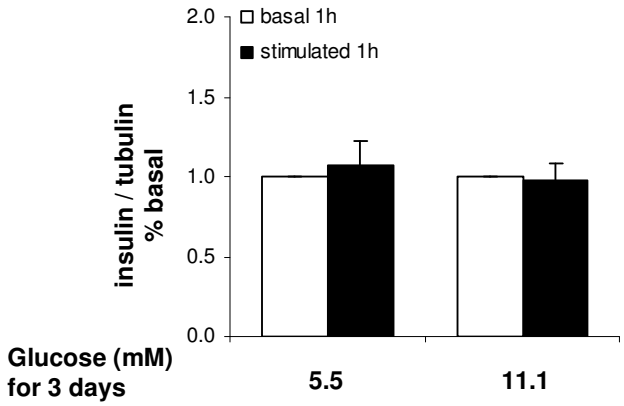

Figure 6: 1-hour glucose stimulation does not result in increased mature insulin mRNA expression.
Total RNA was isolated from human islets that were cultured for 72 hours in 5.5 or 11.1 mM glucose, followed by a 1-hour glucose challenge at 5.5 (basal) or 33.3 mM (stimulated) glucose. In the LightCycler system, quantitative RT-PCR was performed for stimulated insulin gene expression relative to basal gene expression and insulin levels were normalized to tubulin. Data show mean ± SE of four experiments from four organ donors.

5.5 DISCUSSION

The adverse effects that chronic hyperglycemia might have on β-cells have been well described in regard to function and mass. In the present study, we idenfity the MAP-kinases ERK1/2 as keyplayer in the process of glucose desensitization. Supporting the concept that the ERK1/2 cascade is a significant regulator of glucose-dependent events, we demonstrate that β-cell responsiveness correlates with the ability of glucose to upregulate pERK1/2. After prolonged elevated glucose concentrations, human islets do not respond to an acute glucose challenge as observed by abolished stimulated insulin secretion in parallel to inhibited ERK1/2-activation.

The potent role of potassium channel openers to protect from high glucose-induced impaired function and apoptosis have been addressed *in vitro* and *in vivo* in diabetic patients (10-13). Our data confirm these findings as addition of Diazoxide during the chronic incubation period counteracted glucose-induced β-cell dysfunction. We demonstrate that impaired function paralles diminished glucose-induced ERK1/2 activation, both of which are restored by Diazoxide. In addition, we found that ERK1/2 also mediates the adverse effects of prolonged elevated glucose levels, as ERK1/2-inhibition by PD098059 during the chronic period blocked these effects which resulted in restored stimulated insulin secretion together with acute ERK1/2-activation. The similarities of the effects of Diazoxide and PD098059 suggest that ERK1/2-inhibition induces β-cell rest comparable to Diazoxide. By adding somatostatin to the chronic as well as to the acute medium, we confirmed our hypothesis that the observed effects are due to glucose directly affecting β-cells and therefore independent of secreted insulin.

Glucose desensitization is a temporary state of cellular refractoriness (16) that can be reversed upon restoration of normoglycemia, which stands in contrast to glucotoxicity where β-cell damage is potentially irreversible. In our experimental settings, we found that elevated glucose levels of 11.1 mM for 3 days induces glucose desensitization of human islets, since an additional 2 days at control conditions of 5.5 mM led to their recovery as assessed by restored glucose-stimulated insulin secretion together with ERK1/2-activation. However, rising glucose concentrations to supraphysiological 33.3 mM during the chronic period seems to result in glucotoxicity as the islets did not recover ERK1/2-upregulation, showing that, besides culture time, glucose concentrations also play a crucial role in the outcome.

The fact that we did not observe any acute, detectable changes in insulin mRNA levels is in line with a recent study by Evans-Molina et al. (17). The authors investigated the abundance and half-lives of insulin mRNA and pre-mRNAs and found that only pre-mRNA species that contain intron-2 reflect acute changes since they are relatively rare compared to the others and have a short half-life of ~ 60 minutes. Increase in mature (total) mRNA did not

occur before 48 hours. This seems to be controversial to the findings that acute glucose stimulates insulin gene transcription in an ERK1/2-dependent manner (3; 6). However, these studies were done using an insulin-promoter-luciferase construct. In addition, Evans-Molina et al. confirmed that glucose does rapidly increase insulin gene transcritption as they observed increased H4 acetylation and RNA polymerase II recruitment 30 minutes after glucose stimulation. Nonetheless, measurable increases in pre-mRNA are delayed by 60 minutes and mature mRNA by 48 hours.

In conclusion, these data suggest that glucose-mediated adverse effects are dependent on ERK1/2-activity and imply ERK1/2 as mediator of β-cell responses to short- as well as long-term incubations with elevated glucose. ERK1/2 might therefore be a promising novel therapeutic target to preserve β-cell function in diabetes.

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6. CONCLUSION

Increasing evidence indicates that there is a progressive deterioration in β-cell function and mass underlying the development of type 2 diabetes. Research with isolated islets over the past decade identified glucose itself as key physiological regulator of β-cells. It is not only the main stimulus of insulin secretion but it also modulates β-cell turnover in order to adapt to the body's insulin demand. However, it has also been recognized as one of the main contributors leading to β-cell failure and death. Specifically, chronic stimulation due to prolonged elevated glucose levels are detrimental for β-cell survival and insulin release. These deleterious effects are described with the term 'glucotoxicity' and have important consequences in regard to the occurrence of hyperglycemia in type 2 diabetic patients as well as individuals at risk to develop the disease. It suggests that even slightly elevated blood glucose levels or transient postprandial glycemic excursions might have a negative impact on β-cells. Understanding mechanisms and identifying components involved in glucose-induced β-cell destruction have been a focus in the search for a therapy for diabetes and have also been investigated in the course of this work.

Low levels of circulating inflammatory mediators secreted by the adipose tissue have been associated with obesity and type 2 diabetes, indicating that the immune system is involved in the pathogenesis of this disease. The pro-inflammatory cytokine IL-1β has been characterized as contributer to the steady loss of β-cells as its expression has been detected in pancreata from type 2 diabetic patients but not in control subjects. Indeed, this local upregulation in islets is a result of high glucose-induced production and secretion of IL-1β by β-cell themselves which in turn leads to a decline in mass and function, thereby further disturbing glycemic control and fueling a vivicious cycle. The anti-inflammatory cytokine IL-1Ra has been studied *in vitro* and *in vivo* in regard to its effectivness to inhibit IL-1β-mediated effects. IL-1Ra is a naturally occuring antagonist to IL-1β that competitively binds to the same receptor without activating a signaling cascade. It protects human and rodent islets from glucose- and IL-1β-induced functional impairment and apoptosis and improves glycemic control in patients with type 2 diabetes. Our study aimed to investigate the ability of IL-1Ra to prevent diet-induced diabetes and elucidate underlying mechanisms. 12 weeks of high fat/ high surcrose diet (HFD) in wildtype C57BL/6J-mice significantly raised fasting and fed blood glucose levels, a development that was prevented by concomitant, daily IL-1Ra-administration. Intraperitoneal glucose tolerance tests revealed that IL-1Ra also counteracted HFD-induced impairement of glucose clearance. Our data demonstrate that this is a result of the combination of enhanced insulin secretion together with improved insulin action. Examining pancreatic sections and isolated islets from all four treatment groups showed that IL-1Ra induced proliferation in animals under the normal diet group and prevented HFD-

induced apoptosis in cultured islets. Decreased β-cell function as a result of high fat feeding correlated with decreased insulin mRNA levels, both of which were inhibited in the IL-1Ra-treated HFD-group. We found that reduced insulin gene transcription might be due to HFD-induced nuclear export of the insulin transcription factor PDX-1. Improved β-cell function in animals from the HFD-group that received IL-1Ra is associated with nuclear PDX-1 localization comparable to ND-animals. Importantly, our study confirmed a glucose-/ IL-1β-mediated regulation of PDX-1 shuttling in human islets that is counteracted by IL-1Ra, and identified JNK as intermediate factor, providing new insights into how hyperglycemia may directly affect β-cell function. Besides the immediate effects of IL-1Ra on β-cells, we provide evidence that treatment partly prevents sub-clinical systemic inflammation which in turn might contribute to the benefical outcome in regard to β-cell function. IL-1Ra inhibited HFD-induced increased serum levels of the adipokines leptin and resistin as well as of free fatty acids, triglycerides and cholesterol. Analysis of adipose tissue revealed decreased macrophage infiltration and activation as compared to untreated HFD-animals. Our data support the findings that IL-1β plays a critical role in the development of type 2 diabetes and further examines the potency of IL-1Ra as therapy for this disease.

Research has revealed several extra- and intracellular factors that mediate the dual role of glucose on β-cell function and survival and whose precise and directed modulation might switch a toxic signal into a benefical outcome. Among these factors are the protein-kinases ERK1/2. Acute glucose-induced activation leads to insulin gene transcription and insulin secretion, both processes that are partly abolished when ERK1/2-activity is blocked. In contrast, prolonged ERK1/2 phosphorylation is responsible for adverse effects including impaired function and apoptosis. The results of the second project demonstrate that hyperglycemia-induced impaired glucose-stimulated insulin secretion correlates with diminished phospho-ERK1/2 upregulation. Inhibiting overstimulation during chronic incubations restores glucose-induced ERK1/2-activation together with β-cell function. Interestingly, β-cell rest can thereby be achieved by preventing insulin secretion as well as by inhibiting prolonged ERK1/2-activity. Hence, ERK1/2 function as mediators of β-cell responses to short- as well as long-term stimulation with glucose and constitutes a possible target for therapeutic interventions that would switch a toxic into a benefical outcome.

Hyperglycemia, which is the biochemical hallmark of type 2 diabetes, mainly results from a defect in the appropriateness of insulin secretion that occurs together with insulin resistance. Increasing evidence suggests that an impairement of β-cell function precedes diabetes by several years. The progressive decline in β-cell performance in turn may partly be attributed to prevailing elevated blood glucose concentrations. Hence, therapeutic interventions, which are designed to prevent hyperglycemia-induced alterations in β-cells, are desired since they are considered to have a high impact on the survival of functional β-

cells. The findings of the present work underscore the importance of two factors, IL-1Ra and ERK1/2, by elucidating their role in mediating and modulating the adverse effects of glucose and by characterizing their potential as therapeutic targets.

7.1 FURTHER PUBLISHED STUDIES WITH MY CONTRIBUTION AND CO-AUTHORSHIP

7.1.1 Low Concentrations of IL-1β Induces FLIP-Mediated β-Cell Proliferation in Human Pancreatic Islets.

Maedler K, Schuman DM, Sauter N, Ellingsgaard H, Bosco D, Baertschiger R, Iwakura Y, Oberholzer J, Wollheim CB, Gauthier BR, Donath MY.

Diabetes. 2006 Oct;55(10):2713-22

Original Article

Low Concentration of Interleukin-1β Induces FLICE-Inhibitory Protein–Mediated β-Cell Proliferation in Human Pancreatic Islets

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High glucose concentrations have a dual effect on β-cell turnover, inducing proliferation in the short-term and apoptosis in the long-term. Hyperglycemia leads to β-cell production of interleuking (IL)-1β in human pancreatic islets. Fas, a death receptor regulated by IL-1β, is involved in glucose-induced β-cell apoptosis. Fas engagement can be switched from death signal to induction of proliferation when the caspase 8 inhibitor, FLICE-inhibitory protein (FLIP), is active. Here, we show that IL-1β at low concentrations may participate in the mitogenic actions of glucose through the Fas-FLIP pathway. Thus, exposure of human islets to low IL-1β concentrations (0.01–0.02 ng/ml) stimulated proliferation and decreased apoptosis, whereas increasing amounts of IL-1β (2–5 ng/ml) had the reverse effects. A similarly bimodal induction of FLIP, pancreatic duodenal homeobox (PDX)-1, and Pax4 mRNA expression, as well as glucose-stimulated insulin secretion, was observed. In contrast, Fas induction by IL-1β was monophasic. Low IL-1β also induced the IL-1 receptor antagonist (IL-1Ra), suppression of which by RNA interference abrogated the beneficial effects of low IL-1β. The Fas antagonistic antibody ZB4 and small interfering RNA to FLIP prevented low IL-1β–stimulated β-cell proliferation. Consistent with our in vitro results, IL-1β knockout mice displayed glucose intolerance along with a decrease in islet Fas, FLIP, Pax4, and PDX-1 transcripts. These findings indicate that low IL-1β levels positively influence β-cell function and turnover through the Fas-FLIP pathway and that IL-1Ra production prevents harmful effects of high IL-1β concentrations. *Diabetes* 55:2713–2722, 2006

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FLIP, FLICE-inhibitory protein; GSIS, glucose-stimulated insulin secretion; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; IRS, insulin receptor substrate; KRBB, Krebs-Ringer bicarbonate buffer; PDX, pancreatic duodenal homeobox; rh, recombinant human; siFLIP, siRNA directed to FLIP; siIL-1Ra, siRNA directed to IL-1Ra; siRNA, small interfering RNA; TUNEL, transferase-mediated dUTP nick-end labeling.

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The capacity of the pancreatic β-cell to adaptively increase insulin secretion in response to long-term insulin resistance (for example in obesity) is fundamental to the maintenance of normoglycemia. Failure of such a response results in diabetes (1–8). Changes in glucose concentration are key regulators of β-cell secretory function. Short-term exposure of human islets to increased glucose concentrations will enhance insulin production and β-cell proliferation, while prolonged exposure will have toxic effects leading to impaired insulin secretion and β-cell apoptosis (9–17). Various mechanisms for this glucotoxic effect have been proposed, including advanced glycation end products, reactive oxygen species, impairment of insulin gene transcription, and endoplasmic reticulum stress. Recently, interleukin (IL)-1β was shown to be induced in β-cells of patients with type 2 diabetes, indicating that the cytokine could be an important mediator contributing to glucotoxicity (18). Consistent with the latter, β-cell expression of IL-1β correlates with appearance of hyperglycemia in several animal models of diabetes, including the Psammomys obesus, the OLEFT rat, the GK rat, and the human islet amyloid polypeptide transgenic rat ([18–21] and P.C. Butler, personal communication). IL-1β has been shown to impair insulin release and to induce Fas expression enabling Fas-triggered apoptosis in rodent and human islets (16,22–31). Accordingly, increased glucose concentrations also induce β-cell expression of Fas in vitro and in vivo, which will then be activated by the endogenous Fas ligand (16,28,32,33).

Interestingly, the beneficial short-term effects of high glucose on β-cell function and proliferation are also partly mediated by Fas. Indeed, in the presence of the caspase 8 inhibitor, FLICE-inhibitory protein (FLIP), Fas signaling switches from apoptosis to cell replication (34). Furthermore, we recently demonstrated an additional role for the Fas pathway in regulating insulin production and release (35). Additionally, low concentrations of IL-1β stimulate insulin release in rat islets (36). Since glucose induces IL-1β, we hypothesized that IL-1β may also mediate beneficial effects of glucose. We show that low concentrations of IL-1β induce β-cell proliferation and enhance β-cell secretory function via the Fas-FLIP pathway, an effect facilitated by the concomitant IL-1 receptor agonist (IL-1Ra) production.

RESEARCH DESIGN AND METHODS

Ethical approval for mouse studies was granted by the Zurich Cantonal Animal Experimentation Committee. C57BL/6j wild-type mice were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-1β knockout (IL-1β$^{-/-}$) mice, on a C57BL/6j background, were produced by gene targeting as previously described (37). Animals were housed at 22°C with a 12-h light-dark cycle (lights on at 0700) and allowed free access to water and food.

Intraperitoneal glucose and insulin tolerance tests. Mice were fasted 12 h overnight and injected intraperitoneally with 2 mg/g body wt glucose (40% glucose solution; Laboratorium Dr. G. Bichsel AG, Interlaken, Switzerland) or with 0.75 mU/g recombinant human (rh) insulin (Novo Nordisk, Bagsværd, Denmark) for the glucose or insulin tolerance tests, respectively. Blood samples were obtained from tail-tip bleedings, and blood glucose concentration was measured with a Glucometer (Freestyle, Disetronic Medical Systems, Burgdorf, Switzerland).

Islet isolation and culture. Human islets were isolated from pancreata of nine organ donors at the University of Geneva Medical Center and at the University of Illinois at Chicago. Mouse islets were isolated as previously described (38). The islets were cultured on extracellular matrix–coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel), allowing the cells to attach to the dishes and spread. Human islets were cultured in CMRL 1066 medium containing 5.5 mmol/l glucose and mouse islets in RPMI-1640 medium containing 11.1 mmol/l glucose, both supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Invitrogen, Carlsbad, CA), hereafter referred to as culture medium. Two days after plating, when most islets were attached and began to flatten, the medium was changed to culture medium containing 5.5, 11.1, or 33.3 mmol/l glucose with or without 0.01–5 ng/ml rhIL-1β, 500 ng/ml rhIL-1Ra (R&D Systems, Minneapolis, MN), 500 ng/ml antagonistic Fas antibody (ZB4; MBL, Nogoya, Japan), or transfected as described below.

RNA interference. RNAs of 21 nucleotides, designed to target human IL-1Ra (5′AUCUGCAGAGGCCUCCGCAtt3′/5′UGCGGAGGCCUCUGCAGAUtt3′), human FLIP$_{long}$ (silencer predesigned small interfering RNA [siRNA]), and scrambled siRNA were synthesized by Ambion (Austin, TX). siRNA was transfected using SiPortAmine and transfection efficiency estimated with cy3-labeled siRNA using a Silence siRNA Labeling Kit (Ambion), as previously described (39).

β-Cell replication and apoptosis. For β-cell proliferation studies, a monoclonal antibody against the human (Zymed Laboratories, San Francisco, CA) or mouse (Santa Cruz Biotechnology, Santa Cruz, CA) Ki-67 antigen was used. The free 3′-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Boehringer, Mannheim, Germany) and as previously described in detail (14,18). Thereafter, islets were incubated for 30 min at 37°C with a guinea pig anti-insulin antibody (Dako, Carpinteria, CA), followed by detection using the streptavidin-biotin-peroxidase complex (Zymed) or a fluorescein-conjugated rabbit anti–guinea pig antibody (Dako).

Histochemical analysis. The pancreata were weighed, fixed in formalin, and embedded in paraffin. Ten representative sections from each pancreas (spanning the width of the pancreas) were used in the analysis of β-cell mass. Tissue sections were deparaffinized, rehydrated, and incubated with guinea pig anti-insulin antibody (Dako) followed by detection with a fluorescein-conjugated rabbit anti–guinea pig antibody (Dako). Subsequently, the specimens were labeled for glucagon with mouse anti-glucagon antibody (Dako), followed by detection with donkey anti-mouse Cy3-conjugated antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). β-Cell mass was analyzed using Openlab software. The relative area of β-cells (green fluorescence) was determined by quantification of the cross-sectional β-cell area divided by the cross-sectional area of total tissue. The β-cell mass per pancreas was estimated as the product of the relative cross-sectional area of β-cells per total tissue and the weight of the pancreas. For detection of FLIP expression in β-cells, islets were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, incubated with rabbit anti-FLIP (R&D Systems), followed by detection with donkey anti-rabbit Cy3-conjugated antibody (Jackson). Subsequently, the specimens were stained for insulin as described above.

Western blot analysis. Equivalent amounts of protein from each treatment group were run on 15% SDS polyacrylamide gels. Proteins were electrically transferred to nitrocellulose filters and incubated with rabbit anti–caspase 8 (Stressgen, Victoria, BC, Canada), rabbit anti-human FLIP-long (R&D Systems), rabbit anti–pancreatic duodenal homeobox (PDX)-1 (kindly provided by Stefan Zahn; NovoNordisk, Bagsværd, Denmark), or rabbit anti-actin (Cell Signaling, Beverly, MA) antibodies followed by incubation with horseradish peroxidase–linked anti-rabbit IgG peroxidase-conjugated antibodies (Santa Cruz Biotechnology). Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signaling).

RNA extraction and quantitative RT-PCR. Total RNA was extracted from the cultured islets by using the RNeasy Mini Kit (Qiagen, Basel, Switzerland), and RT-PCR was performed by using the SuperScript Double-Stranded cDNA Synthesis Kit according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). For quantitative analysis, we used the LightCycler Quantitative PCR System (Roche, Basel, Switzerland) with a commercial kit (LightCycler-DNA Master SYBR Green I; Roche). Mouse primers used were 5′TACGGGGTTTGTGAAAGGAG3′ and 5′CACATCATTCCCCAGGAAAC3′ (insulin), 5′GAGGACCCGTACAGCCTACA3′ and 5′CGTTGTCCCGCTACTAC GTT3′ (PDX-1), 5′CTAAATTTGGTTGCCCCAGA3′ and 5′CTCCCATTATG GAGCCTGAA3′ (FLIP long), 5′GCTCTTTTTGCCTGGGAGATC3′ and 5′CCCG AAGGACTCGATTGATAGA3′ (Pax4), 5′TATCAAGGAGGCCCATTTTG3′ and 5′GGTCAGGGTGCAGTTTGTTT (Fas), and 5′GTGGCAGTGATGGCATGG AC3′ and 5′CAGCACCAGTGGATGCAGGG3′ (glyceraldehyde-3-phosphate dehydrogenase). Human primers used were 5′CCACCTTGGGACCTGTTTAG3′ and 5′TGATGCCAGAGGAAGAGGAG3′ (PDX-1), 5′CCACCGGAATCGGAC TATCTT3′ and 5′TACTGCCCACGCTGGAACTC3′ (Pax4), 5′GAGCAAGC CCCTAGGAATCT3′ and 5′GCCCTGAGTGAGTCTGATCC3′ (FLIP long), 5′CTACCTAGTGTGCGGGGAAC3′ and 5′GCTGGTAGAGGGAGCAGATG3′ (insulin), 5′GCATCTGGACCCTCCTACCT3′ and 5′CAGTCTGGTTCATCCC CATT3′ (Fas), 5′AGAGTCGCGCTGTAAGAAGC3′ and 5′TGGTCTTGTCACT TGGCATC3′ (α-tubulin), 5′AACAGCGACACCCACTCCTC3′ and 5′GGAGG GGAGATTCAGTGTGGT3′ (glyceraldehyde-3-phosphate dehydrogenase), and 5′TACGGGTCCTGGCATCTTGT3′ and 5′CCATTTGTGTTGGGTCCAGC3′ (cyclophilin).

Insulin and IL-1Ra release and insulin content. For acute insulin release in response to glucose, islets were washed and preincubated (30 min) in Krebs-Ringer bicarbonate buffer (KRBB) containing 2.8 mmol/l glucose and 0.5% BSA. KRBB was then replaced by KRBB 2.8 mmol/l glucose for 1 h (basal), followed by an additional 1 h in KRBB 16.7 mmol/l glucose. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. To determine the total insulin content of the pancreas, the tissue was homogenized in 1 ml 0.18 N HCl in 70% ethanol and left overnight at 4°C. Insulin was determined using a human insulin radioimmunoassay kit (CIS Bio International, Gif-Sur-Yvette, France), which has similar affinity for both mouse and human insulin. Serum insulin was measured using Luminex technology according to the manufacturer's instructions (Linco Research, St. Charles, MO). IL-1Ra release in the islet supernatant was measured by using human anti–IL-1Ra enzyme-linked immunosorbent assay kits (R&D).

Statistical analysis. Samples were evaluated in a randomized manner by a single investigator (K.M.) who was blinded to the treatment conditions. Data are presented as means ± SE and were analyzed by Student's t test or by ANOVA with a Bonferroni correction for multiple-group comparisons.

RESULTS

Low concentrations of IL-1β induce β-cell proliferation, are antiapoptotic, and enhance β-cell secretory function. Human islets were cultured on extracellular matrix–coated plates in the presence of increasing IL1-β concentrations for 4 days. Exposure to 0.02 ng/ml IL-1β induced a twofold increase in β-cell proliferation compared with controls, but exposure to higher concentrations of 2 and 5 ng/ml resulted in an ~1.5-fold decrease (Fig. 1*A* and *B*; Table 1). By contrast, 0.01 ng/ml IL-1β reduced baseline β-cell apoptosis twofold, whereas higher doses of 2 and 5 ng/ml IL-1β increased the apoptosis rate by 2.3- and 3.6-fold, respectively (Fig. 1*A* and *C*; Table 1). Those changes in β-cell turnover were accompanied by a 1.6-fold increase in glucose-stimulated insulin secretion (GSIS) at 0.02 ng/ml IL-1β and a 2.8-fold reduction by 2 ng/ml IL-1β (Fig. 1*D* and *E*). Insulin content of the islets was not significantly affected (data not shown).

Endo- and exogenous modulation of IL-1 signaling by IL-1Ra. We have recently shown expression of IL-1Ra in human β-cells (39). Interestingly, secreted IL-1Ra will antagonize the effect of IL-1β. To highlight the potential cross-talk between these two molecules, we repressed endogenous production IL-1Ra by RNA interference. As previously shown, siRNA directed to IL-1Ra (siIL-1Ra) with a transfection efficiency of ~70% suppressed IL-1Ra

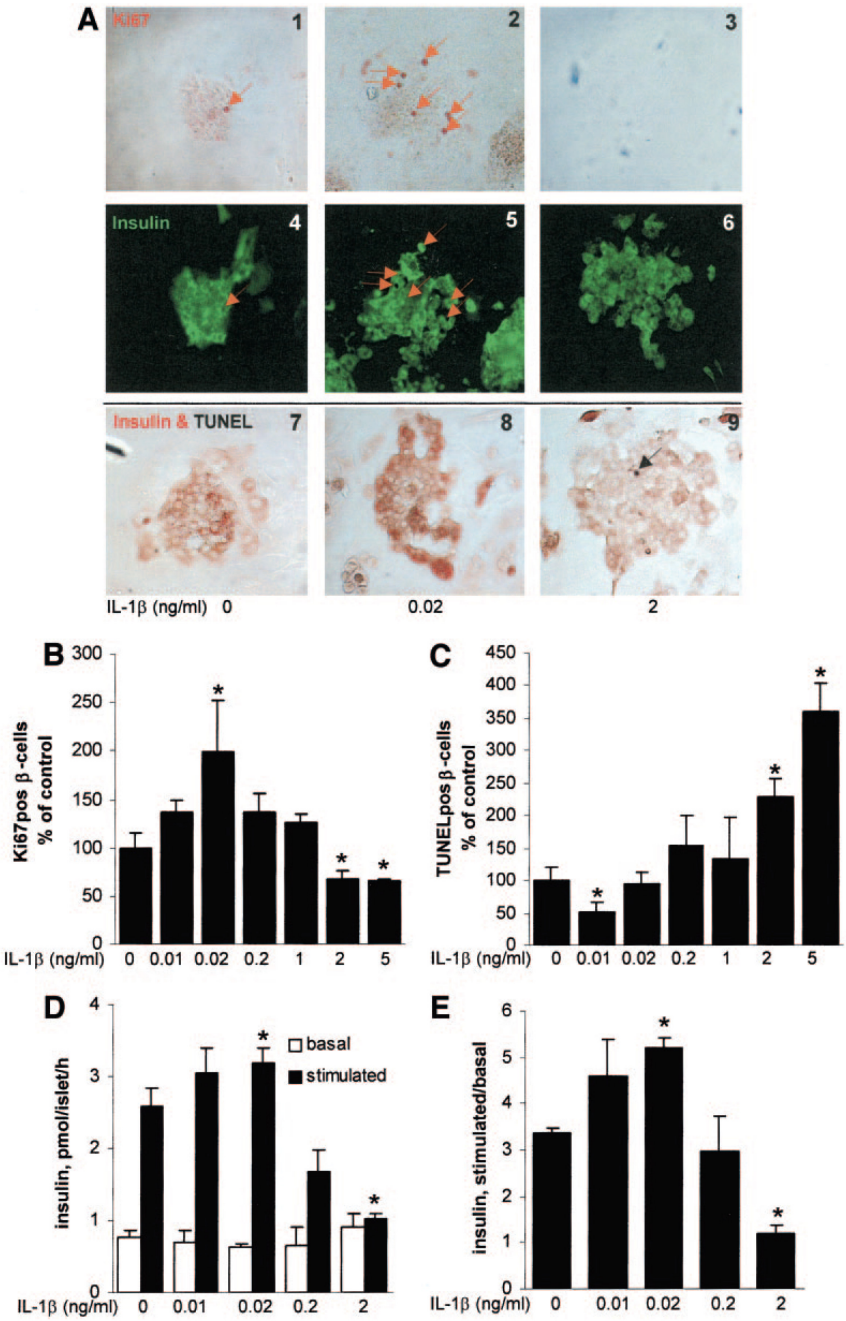

FIG. 1. Low concentrations of IL-1β induce β-cell proliferation, are antiapoptotic, and enhance β-cell secretory function. Human islets were cultured on extracellular matrix–coated dishes for 4 days at 5.5 mmol/l glucose with increasing concentrations of IL-1β. Double immunostaining for β-cell proliferation with anti–Ki-67 in brown (*A1–3*) and anti-insulin in green (*A4–6*) and for β-cell apoptosis with the TUNEL assay in black and anti-insulin in brown (*A7–9*), in control islets (*A1*, *4*, and *7*), and in islets treated with 0.02 (*A2*, *5*, and *8*) and with 2 (*A3*, *6*, and *9*) ng/ml IL-1β. The orange arrows mark β-cells stained positive for Ki-67 and insulin, and the black arrow marks a TUNEL-positive β-cell. Percentage of Ki-67–positive (*B*) and TUNEL-positive (*C*) β-cells normalized to control incubations at 5.5 mmol/l glucose alone (100%; in absolute values: 0.19 ± 0.07% Ki-67–positive β-cells and 0.4 ± 0.05% TUNEL-positive β-cells). The mean number of islets scored was 41 (*B*) and 52 (*C*) for each treatment condition from each donor. Basal and GSIS denote the amount secreted during successive 1-h incubations at 2.8 (basal) and 16.7 (stimulated) mmol/l glucose following the 4-day culture period expressed as secreted insulin (*D*) or stimulatory index (*E*). Islets were isolated from four organ donors. Results are means ± SE. *$P < 0.05$ to untreated controls.

TABLE 1
TUNEL and Ki-67 labeling of β-cell nuclei

IL-1β (ng/ml)	TUNEL			Ki-67		
	Number of cells counted	Number of TUNEL-labeled nuclei	Percent of TUNEL-labeled nuclei	Number of cells counted	Number of Ki-67–labeled nuclei	Percent of Ki-67–labeled nuclei
0	14,800	57	0.3851	21,300	39	0.1831
0.01	8,500	15	0.1765	10,100	20	0.1980
0.02	16,400	50	0.3049	18,600	63	0.3387
0.2	16,900	79	0.4675	18,000	39	0.2167
1	8,100	30	0.3704	10,500	17	0.1619
2	15,500	132	0.8516	22,100	30	0.1357
5	16,900	215	1.2722	19,800	22	0.1111

Human islets were cultured on extracellular matrix–coated dishes for 4 days at 5.5 mmol/l glucose with increasing concentrations of IL-1β and double stained for β-cell apoptosis with the TUNEL assay and for proliferation with anti–Ki-67. Islets were isolated from four organ donors.

mRNA by 69 ± 6% (39). Blockade of endogenous IL-1Ra by siIL-1Ra did not significantly affect baseline β-cell proliferation (Fig. 2*A*) but led to a twofold increase in apoptosis, levels similar to the ones obtained with 2 ng/ml IL-1β (Fig. 2*B*). Addition of 0.02 ng/ml IL-1β failed to increase β-cell proliferation and to further increase β-cell apoptosis (Fig. 2*A* and *B*). In contrast, addition of exogenous rhIL-1Ra (500 ng/ml) increased β-cell proliferation and protected from the deleterious effects of a high concentration of 2 ng/ml IL-1β. Furthermore, the compound protected cells against prolonged exposure to 33.3 mmol/l glucose (Fig. 2*A* and *B*). Similarly, siIL-1Ra completely blocked GSIS in the absence or presence of 0.02 ng/ml IL-1β, whereas exogenous rhIL-1Ra prevented the impairment of GSIS in the presence of 2 ng/ml IL-1β or 33.3 mmol/l glucose (Fig. 2*C* and *D*). Interestingly, the low concentration of 0.02 ng/ml IL-1β induced a 1.8-fold increase in the release of its receptor antagonist IL-1Ra into the culture medium (Fig. 2*E*).

Low-dose IL-1β–induced β-cell proliferation is mediated via the Fas-FLIP pathway and may involve PDX-1 and Pax4. Next, we investigated the underlying mechanisms of the proliferative effect of IL-1β. We hypothesized that the Fas pathway mediates IL-1β–induced β-cell proliferation. To test this hypothesis, first we investigated whether low concentrations of IL-1β are capable of inducing Fas. Exposure of human islets to 0.02 ng/ml IL-1β for 4 days induced Fas expression to levels similar to those observed with higher concentrations of IL-1β and 33.3 mmol/l glucose (Fig. 3*A*). However, low concentrations of IL-1β did not activate caspase 8, the most upstream caspase in the Fas apoptotic pathway, while higher concentrations than 0.02 ng/ml IL-1β cleaved procaspase 8, releasing the active form of the protease (Fig. 3*B*). To examine whether the induction of proliferation by 0.02 ng/ml IL-1β is caused by the interaction of constitutively expressed Fas ligand (16,28) and upregulated Fas, we used the Fas antagonistic antibody ZB4. ZB4 inhibited the proliferative effect of IL-1β (Fig. 3*C*). Since Fas signaling may induce proliferation in the presence of FLIP (34), we analyzed FLIP protein modulation by increasing concentrations of IL-1β. Similar to IL-1β–mediated changes in β-cell proliferation (Fig. 1*B*), low concentrations of IL-1β induced FLIP expression, while higher concentrations were inhibitory (Fig. 3*D* and *E*). Of note, FLIP mRNA expression remained unchanged (data not shown) in agreement with previous studies showing regulation of FLIP only at the protein level (34). The functional role of FLIP in IL-1β–induced β-cell proliferation was then investigated by RNA interference (siRNA directed to FLIP [siFLIP]). siFLIP suppressed FLIP expression in most β-cells (Fig. 3*F*) leading to a 5.2-fold decrease in FLIP protein expression (for representative blot, see Fig. 3*G*). Repression of FLIP resulted in impaired baseline β-cell proliferation and prevented IL-1β–mediated replication, whereas scrambled siRNA had no effect on the number of Ki-67–positive β-cells (Fig. 3*H*). We next investigated the potential implication of the transcription factors PDX-1 and Pax4, known to promote β-cell replication (40–45). Low concentrations of IL-1β stimulated PDX-1 mRNA and protein expression, which were decreased by higher concentrations of IL-1β (Fig. 3*I–K*). Similarly, low concentrations of IL-1β stimulated Pax4 and insulin mRNA expression, which were decreased by higher concentrations (Fig. 3*L* and *M*). However, the stimulation of insulin mRNA by low IL-1β failed to reach statistical significance. Furthermore, Pax4 was stimulated at 12 h but no longer after 4 days.

Impaired glucose tolerance in IL-1β knockout mice. To substantiate the role of IL-1β in normal glucose homeostasis in vivo, glucose tolerance tests were performed in IL-1β knockout (IL-1β$^{-/-}$) mice. As predicted, we found that IL-1β$^{-/-}$ mice displayed glucose intolerance (Fig. 4*A*). Normal sensitivity of the IL-1β$^{-/-}$ mice to injected insulin (Fig. 4*B*) ruled out the possibility that insulin resistance was responsible for the impaired glucose tolerance. Food intake and body weight were similar in IL-1β$^{-/-}$ and wild-type mice (not shown). Analysis of pancreatic tissue of IL-1β$^{-/-}$ mice revealed a normal structure of the islets with no significant decrease in β-cell mass (Fig. 4*C* and *D*). However, insulin mRNA expression was strongly decreased (Fig. 4*E*), although pancreatic insulin content showed no significant changes (5.05 ± 1.04 vs. 4.0 ± 0.7 mol/g insulin per pancreas in normal versus IL-1β$^{-/-}$ mice, respectively). Consistent with our in vitro experiments, Fas, FLIP, PDX-1, and Pax4 were decreased in IL-1β$^{-/-}$ mice (Fig. 4*E*). Islets of IL-1β$^{-/-}$ mice exhibited a strong decrease in baseline β-cell proliferation and GSIS compared with wild-type mice, while apoptosis and insulin content were not significantly different (Fig. 5).

The resistance of IL-1β$^{-/-}$ islets to β-cell glucotoxicity was then studied. Chronic exposure of wild-type mouse islets to 33.3 mmol/l glucose for 4 days impaired β-cell proliferation, induced apoptosis, impaired GSIS, and decreased insulin content (Fig. 5). In contrast, islets of IL-1β$^{-/-}$ mice were not further altered by high glucose

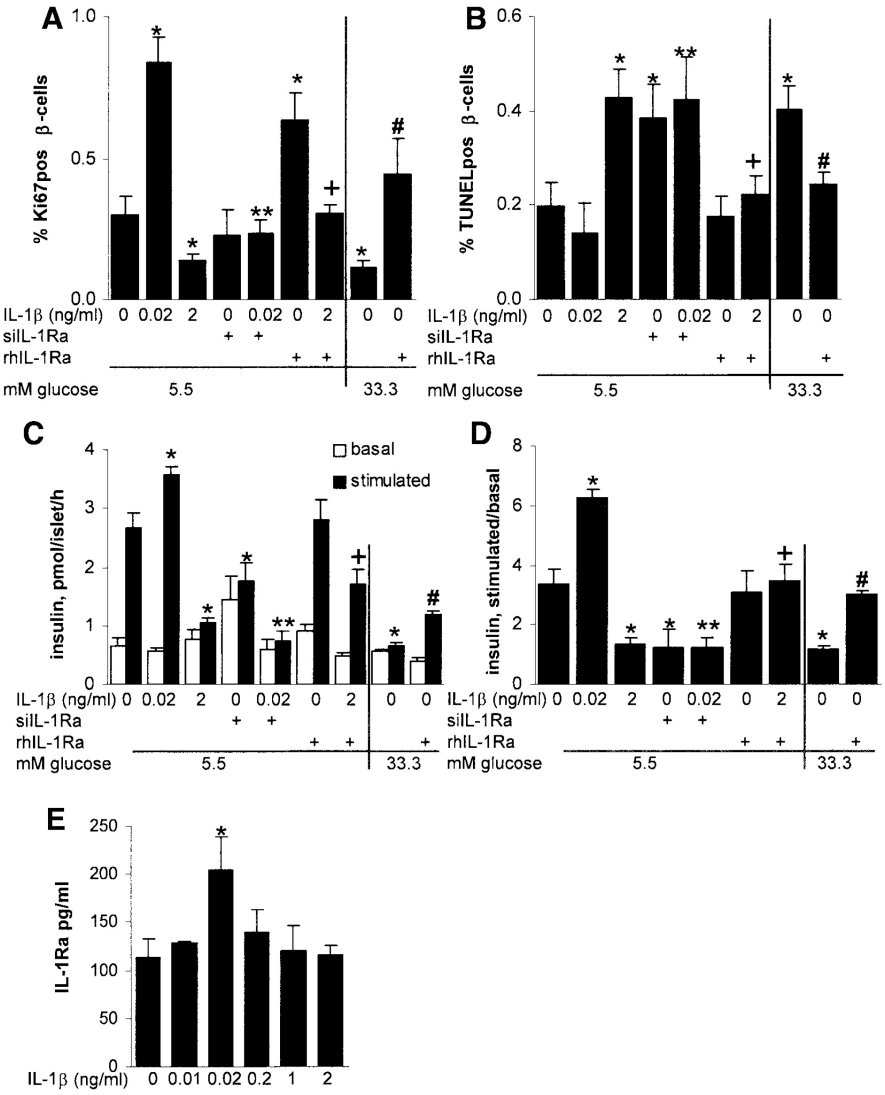

FIG. 2. Endo- and exogenous modulation of IL-1 signaling by IL-1Ra. Human islets were cultured on extracellular matrix–coated dishes for 4 days at 5.5 or 33.3 mmol/l glucose alone or with increasing concentrations of IL-1β or after transfection with 50 nmol/l siIL-1Ra or with addition of 500 ng/ml exogenous rhIL-1Ra. Results are means ± SE of percentage of Ki-67–positive (*A*) and TUNEL-positive (*B*) β-cells. The mean number of islets scored was 45 (*A*) and 43 (*B*) for each treatment condition from each donor. Basal and GSIS denote the amount secreted during successive 1-h incubations at 2.8 (basal) and 16.7 (stimulated) mmol/l glucose following the 4-day culture period expressed as secreted insulin (*C*) or stimulatory index (*D*). *E*: Secretion of IL-1Ra from human islets during 4 days of culture. Data were collected from three tubes per treatment in five separate experiments from five donors. Results are means ± SE. **P* < 0.05 to untreated control. ***P* < 0.05–0.02 ng/ml IL-1β alone. +*P* < 0.05–2 ng/ml IL-1β alone. #*P* < 0.05–33 mmol/l glucose alone.

concentrations (Fig. 5), supporting the concept of IL-1β–mediated glucotoxicity (18). Finally, we have confirmed that glucose regulates IL-1β secretion in mouse islets (2.85 ± 0.8 and 5.68 ± 1.6 pg/ml of IL-1β in islets cultured for 4 days in 11.1 and 33.3 mmol/l glucose, respectively, $n = 5$) as described in human islets (18).

DISCUSSION

The role of IL-1β and other cytokines in the pathogenesis of type 1 diabetes is well established (25,46). More recently, the concept emerged that cytokines may also mediate nutrient-induced β-cell dysfunction during the development of type 2 diabetes (5,6,47,48). Intriguingly, some of these cytokines can be produced by β-cells, including IL-6, IL-1β, IL-1Ra, and pancreatic-derived factor (18,19,39,49–53). The fact that the nonimmune β-cell synthesizes cytokines suggests their implication in islet physiology. Accordingly, we observed beneficial effects of low concentrations of IL-1β on β-cell proliferation, apoptosis, and secretory function.

A low concentration of IL-1β stimulated IL-1Ra, which in turn stimulated β-cell proliferation. Likewise, adenovi-

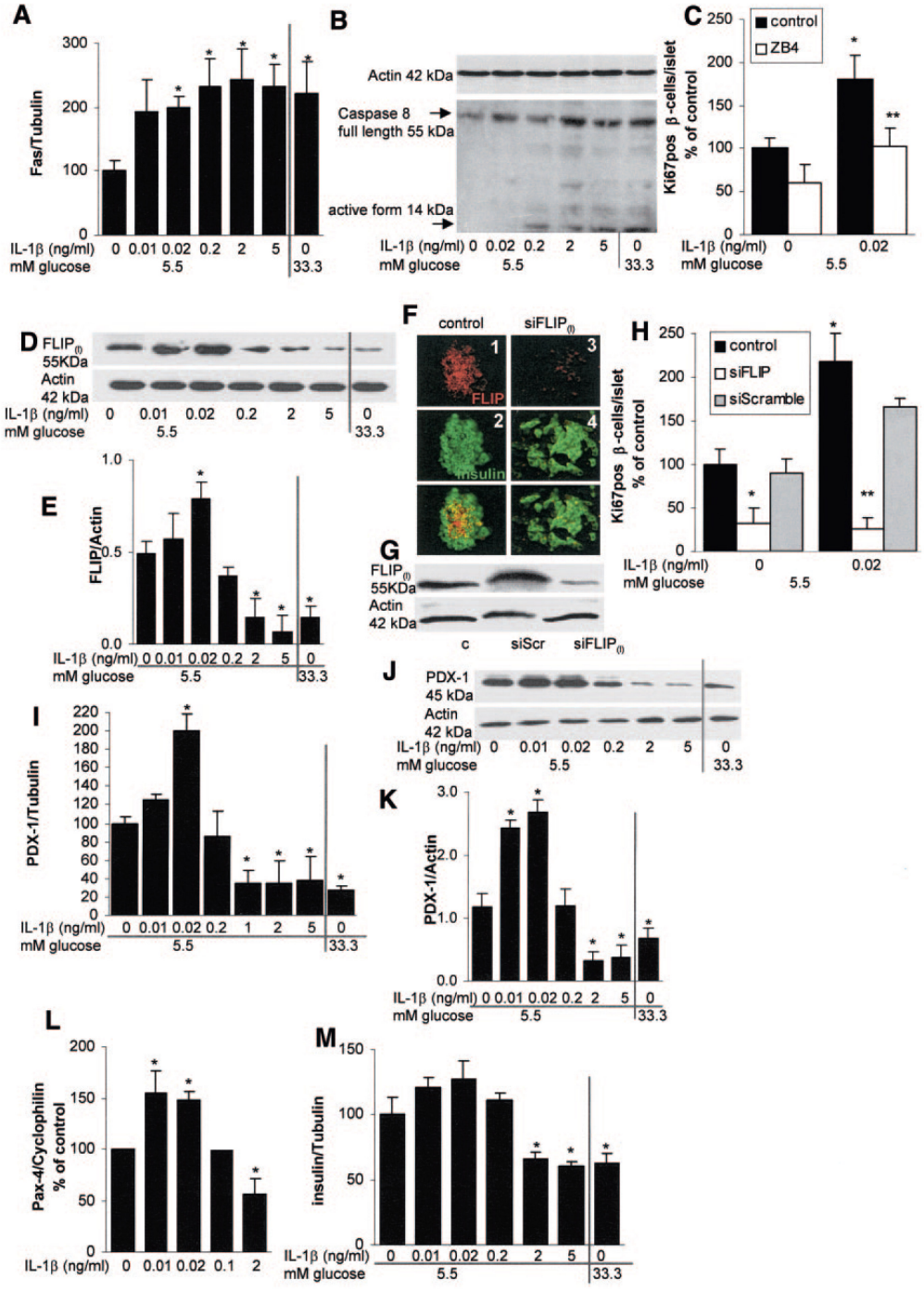

FIG. 3. Low-dose IL-1β–induced β-cell proliferation is mediated via the Fas-FLIP pathway and involves PDX-1 and Pax4. Human islets were cultured on extracellular matrix–coated dishes and exposed for 4 days to increasing IL-1β concentrations or 33.3 mmol/l glucose or 500 ng/ml of the antagonistic anti-Fas antibody ZB4 or 50 nmol/l siRNA to $FLIP_{(long)}$ [$siFLIP_{(l)}$] or to scrambled siRNA (siScr). *A*: Quantitative RT-PCR analysis of Fas expression. *B*: Representative immunoblotting of caspase 8 and actin. For detection of caspase 8, an antibody recognizing full-length (procaspase 8; 55 kDa) and the 14 kDa processed active form of caspase 8 was used. *C*: β-Cell proliferation determined by double staining with anti–Ki-67 and anti-insulin. *D*: Representative immunobloting of $FLIP_{(L)}$ and actin. *E*: The density of expression levels were quantified after scanning and normalized to actin levels. *F*: Double immunostaining of the islets with anti-$FLIP_{(L)}$ (*1* and *3*) and anti-insulin (*2* and *4*) antibodies

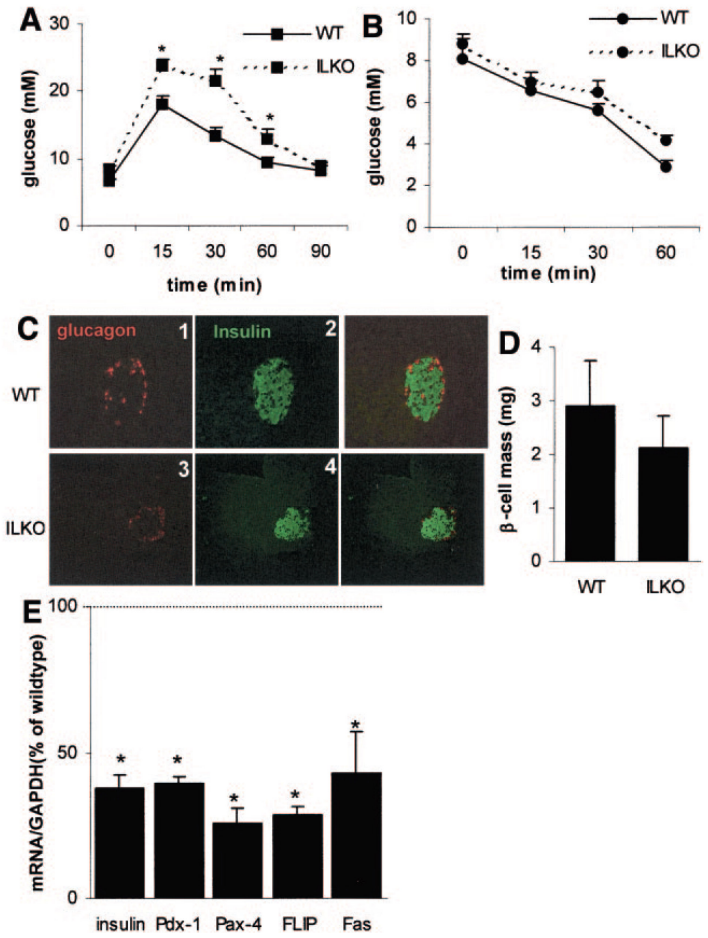

FIG. 4. Impaired glucose tolerance in IL-1β knockout mice. Blood glucose levels following intraperitoneal injection of glucose (*A*) or insulin (*B*) in 3-month-old male IL-1β$^{-/-}$ (ILKO) and wild-type (WT) C57BL/6j mice. *P < 0.05, ILKO vs. C57BL/6j. Data were collected from three separate experiments, each with five animals per group. Double immunostaining for glucagon in red (*1* and *3*) and insulin in green (*2* and *4*) (*C*) and β-cell mass in tissue sections of 4-month-old male wild-type C57BL/6j (*1* and *2*) and IL-1β$^{-/-}$ mice (*3* and *4*) (*D*); n = 5 for each group. *E*: Quantitative RT-PCR detection of insulin, PDX-1, Pax4, FLIP, and Fas mRNA expression. Total RNA was isolated from IL-1β$^{-/-}$ and C57BL/6j islets after 1 day in culture. The level of mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the results expressed as percentage of wild-type islets mRNA levels; n = 3 for each group of mice islets, each in duplicate. *P < 0.05 to wild-type islets.

ral expression of IL-1Ra increases β-cell replication in rat islets (54). At first sight, these results suggest a signaling function for IL-1Ra. However, this is unlikely since IL-1Ra only has a single IL-1 receptor–binding domain and is therefore unable to recruit the IL-1 receptor accessory protein, the second chain of the receptor complex, which is believed to be necessary for signaling (55). Therefore, the proliferative effects of exogenous IL-1Ra probably result from a restoration of a beneficial ratio of IL-1 to IL-1Ra and not from direct effects of the latter. Corroborating this hypothesis, repression of endogenous IL-1Ra was deleterious, probably due to unbalanced actions of IL-1.

Fas expression on the surface of pancreatic β-cells contributes to cytokine-induced apoptosis (30,56). However, when FLIP is activated, Fas becomes mitogenic (34). We propose that the Fas-FLIP pathway is modulated by IL-1β. This is supported by a simultaneous elevation of Fas and FLIP proteins at low IL-1β concentrations leading to β-cell proliferation. In contrast, higher concentrations of IL-1β decreased FLIP while Fas remained elevated, leading to decreased proliferation and induction of apoptosis. Furthermore, the Fas antagonistic antibody ZB4 and siRNA to FLIP both prevented IL-1β–induced β-cell proliferation.

The physiological importance of IL-1β was also apparent

in control islets (*1* and *2*) and siFLIP$_{(L)}$-treated islets (*3* and *4*). *G*: Representative immunobloting of FLIP$_{(L)}$ and actin. *H*: β-Cell proliferation determined by double staining with anti–Ki-67 and anti-insulin. *I*: Quantitative RT-PCR analysis of PDX-1 expression. *J*: Representative immunobloting of PDX-1 and actin. *K*: The density of expression levels were quantified after scanning and normalized to actin levels. Quantitative RT-PCR analysis of Pax4 in islets exposed for 12 h to IL-1β (*L*) and of insulin in islets exposed for 4 days to IL-1β (*M*). Results are means ± SE. In the LightCycler quantitative PCR system, the levels of Pax4 and insulin expression were normalized against tubulin or cyclophylin and the results were expressed as mRNA levels relative to control incubations. The antibodies were blotted on the same membrane after stripping, and actin was used as loading control. Islets were isolated from six organ donors. *P < 0.05 to untreated control; **P < 0.05–0.02 ng/ml IL-1β.

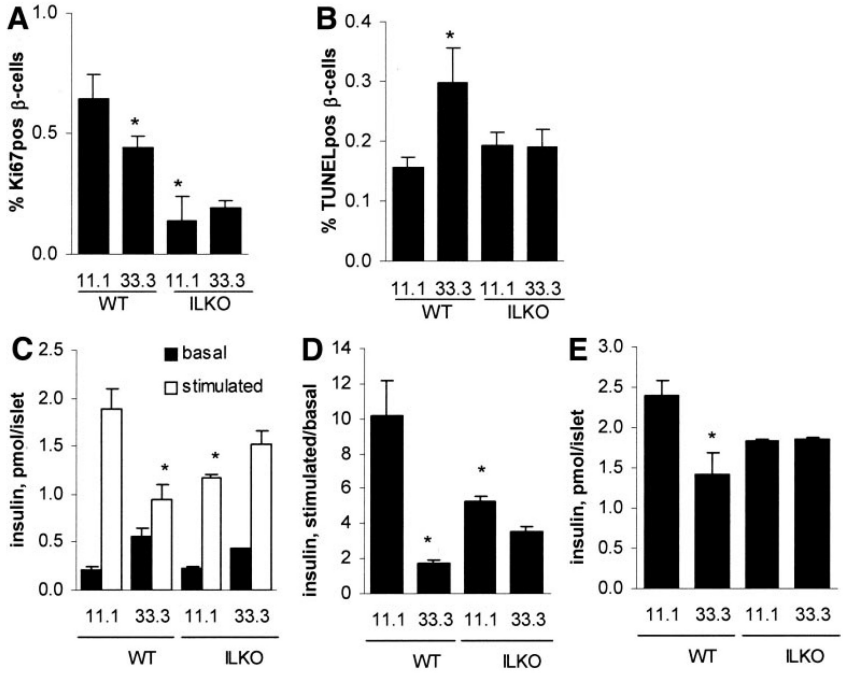

FIG. 5. Resistance of IL-1β$^{-/-}$ islets to glucotoxicity. Isolated islets from male IL-1β$^{-/-}$ (ILKO) and wild-type (WT) C57BL/6j mice were cultured on extracellular matrix–coated dishes for 4 days at 11.1 or 33.3 mmol/l glucose. Results are means ± SE of percentage of Ki-67–positive (*A*) and TUNEL-positive (*B*) β-cells. The mean number of islets scored was 132 (*A*) and 118 (*B*) for each treatment condition in three independent experiments. *C*: Basal and GSIS denote the amount secreted during successive 1-h incubations at 2.8 (basal) and 16.7 (stimulated) mmol/l glucose following the 4-day culture period expressed as secreted insulin (*C*) or stimulatory index (*D*). *E*: Insulin content. Results are means ± SE. Islets were isolated from nine mice for each treatment group in three independent experiments. *$P < 0.05$ to wild-type control.

in IL-1β knockout mice, showing impaired glucose tolerance. Islets of these mice displayed decreased mRNA expression levels of PDX-1, Pax4, insulin, Fas, and FLIP. Of note, these transcripts were enhanced by 0.02 ng/ml IL-1β. At higher concentrations, IL-1β suppressed these factors uncovering its toxic effects. IL-1β may participate in glucotoxicity (18), a concept substantiated by the protection from harmful actions of high glucose of IL-1β knockout mouse islets.

Most factors playing a role in β-cell turnover are also involved in the regulation of β-cell secretory function (57). This is also true for IL-1β, which will have distinct effects depending on the duration of exposure. On the short-term, IL-1β will predominantly influence β-cell secretory function independently of changes in β-cell mass. Indeed, in vitro, a 4-day exposure of human islets to different concentrations of IL-1β led to changes in cell turnover in <1% of β-cells, although β-cell secretory function was almost completely blocked at high concentrations. Nevertheless, this does not mean that prolonged exposure to IL-1β will not affect β-cell mass. Indeed, β-cells remain positive for Ki-67 for ~12 h. In absolute value, 0.02 ng/ml IL-1β increased the number of proliferating β-cells by 0.2%. The human pancreas has between 500,000 and 1,000,000 islets, comprising 500–2,000 β-cells each. Therefore, it can be estimated that low concentrations of IL-1β will increase the number of β-cells by ~4,000,000 per day, leading to a doubling of β-cell mass within 250 days. Such an impressive increase in β-cell mass may occur in vivo over a similar time period (e.g., during obesity [4]). Therefore, the observed differences are within an expected range and support the relevance of the finding.

A key signaling molecule regulating β-cell turnover and function is insulin receptor substrate (IRS)-2 (58). Interestingly, it has been proposed that IL-1β may mediate its deleterious effects via IRS-2 degradation (8). Conversely, it is conceivable that at low concentrations IL-1β also signals via IRS-2. Indeed, while IRS-2 degradation leads to apoptosis, IRS-2 activation enhances β-cell proliferation and function. However, further investigations are required to support this hypothesis.

Cytokines are central in the development of diabetes. However, at low concentrations, IL-1β promotes β-cell function and survival. Therefore, glucose-induced IL-1β may have an important role in the long-term adaptation of the β-cells to hyperglycemia. Short-term exposure to hyperglycemia will induce low levels of IL-1β, inducing IL-1Ra, Fas, and FLIP, leading to decreased apoptosis and enhanced proliferation and function. However, prolonged hyperglycemia will increase the ratio of IL-1β to IL-1Ra, decreasing FLIP and directing Fas to signals deleterious to the cell.

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7.1.2 The Diabetes-Linked Transcription Factor Pax4 is Expressed in Human Pancreatic Islets and is Activated by Mitogens and GLP.

Brun T, Hu He KH, Lupi R, Boehm B, Wojtusciszyn A, Sauter N, Donath M, Marchetti P, Maedler K, Gauthier BR.

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The diabetes-linked transcription factor Pax4 is expressed in human pancreatic islets and is activated by mitogens and GLP-1

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We previously demonstrated that the transcription factor Pax4 is important for β-cell replication and survival in rat islets. Herein, we investigate Pax4 expression in islets of non-diabetic and diabetic donors, its regulation by mitogens, glucose and the incretin GLP-1 and evaluate its effect on human islet proliferation. Pax4 expression was increased in islets derived from Type 2 diabetic donors correlating with hyperglycaemia. *In vitro* studies on non diabetic islets demonstrated that glucose, betacellulin, activin A, GLP-1 and insulin increased Pax4 mRNA levels. Glucose-induced Pax4 expression was abolished by the inhibitors LY294002, PD98050 or H89. Surprisingly, increases in Pax4 expression did not prompt a surge in human islet cell replication. Furthermore, expression of the proliferation marker gene Id2 remained unaltered. Adenoviral-mediated expression of human Pax4 resulted in a small increase in Bcl-xL expression while Id2 transcript levels and cell replication were unchanged in human islets. In contrast, overexpression of mouse Pax4 induced human islet cell proliferation. Treatment of islets with 5-Aza-2′-deoxycytidine induced Pax4 without stimulating Bcl-xL and Id2 expression. Human Pax4 DNA binding activity was found to be lower than that of the mouse homologue. Thus, human *pax4* gene expression is epigenetically regulated and induced by physiological stimuli through the concerted action of multiple signalling pathways. However, it is unable to initiate the transcriptional replication program likely due to post-translational modifications of the protein. The latter highlights fundamental differences between human and rodent islet physiology and emphasizes the importance of validating results obtained with animal models in human tissues.

INTRODUCTION

The ultimate goal in the management of diabetes is to achieve optimal glucose control while avoiding hypoglycaemia. Human islet transplantation has provided proof of principle that it is feasible to partially normalize blood glucose in Type 1 diabetic patients (1). However, this approach is severely hampered by the shortage of donor pancreata and thus alternative sources of cells as well as protocols are required to generate new surrogate β-cells. The encouraging, yet controversial results obtained to date with either embryonic or adult stem cells, necessitate re-evaluation of approaches to be taken in order to produce safe and fully differentiated insulin-producing cells *in vitro* (2). More recently, *in vivo* cell regeneration has gained attention with the finding that residual β-cells are detected in long standing Type 1 diabetic patients (3,4). Consistent with the latter, we and others have proposed that β-cells can replicate and most likely constitute the main venue of cell regeneration under physiological conditions (5–8). Harnessing signals and factors involved in controlling β-cell replication to restore glucose homeostasis in Type 1 diabetic patients would circumvent the need for

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insulin therapy or invasive surgery. Individuals with Type 2 diabetes would also benefit from agents that protect or expand β-cell mass, as several studies have clearly demonstrated a significant decrease in insulin-producing cells in these patients (9,10).

Of particular interest is the β-cell transcription factor Pax4 for which polymorphisms and mutations have been associated with Types 1 and 2 diabetes, respectively (11–17). Consistent with the oncogenic function of other *pax* genes in human cancer (18), we have demonstrated that Pax4 is a key regulator of β-cell plasticity (5). Mitogens such as activin A or betacellulin induced Pax4 expression with a concomitant increase in rat β-cell replication. Overexpression of murine Pax4 in rat as well as in human islets induced β-cell proliferation and conferred protection against cytokine-induced apoptosis. These beneficial effects were conveyed by increased Bcl-xL (an anti-apoptotic gene), c-myc (a proto-oncogene) and Id2 (a c-myc target gene) mRNA levels in rat islets (5). Similarly, the purified recombinant Pax4 protein was recently found to permeate into human cell lines as well as pancreatic islets and subsequently activate Bcl-xL and c-myc (19). Interestingly, low concentrations of IL-1β induced both endogenous Pax4 transcription and β-cell proliferation whereas high levels of the cytokine inhibited expression of the transcription factor and induced apoptosis in human islets (20). The latter findings indicate an initial beneficial effect of cytokines on islet mass whereas higher levels become detrimental, a phenomenon potentially mimicking the *in vivo* conditions of both Types 1 and 2 diabetic patients (21).

Although our previous studies clearly indicate that Pax4 is an important molecular mediator relaying physiological cues to islet mass adaptation, no data on the effects of endogenous Pax4 on human islet cell proliferation are available. Therefore, in the present study, we investigated the expression of Pax4 in islets of non-diabetic and diabetic patients, its regulation by mitogens, glucose and the incretin GLP-1 as well as evaluating its effect on cell replication.

RESULTS

Pax4 is expressed in human pancreatic islets and is increased in Type 2 diabetic patients with BMI between 22 and 26

In order to determine whether Pax4 expression was modulated in pathophysiological conditions such as hyperglycaemia and/or obesity, Pax4 transcript levels were evaluated in islets freshly isolated from a small cohort of Type 2 diabetic donors and related to body mass index (BMI). Pax4 transcript was detected in human islets and increased 10-fold in diabetic donors with a BMI between 22 and 26 when compared with control non-diabetic donors (Fig. 1; 5.7 ± 2.8 versus 0.5 ± 0.1, $P < 0.05$). In contrast to BMI, no correlation between Pax4 mRNA levels and age or sex could be established. Although few donors were analysed, no changes in Pax4 mRNA levels were detected in either group with BMI greater than 26. Noteworthy, control islets exhibited astonishingly small variations in Pax4 mRNA levels independent of BMI. These results suggest that hyperglycaemia is most likely sufficient to induce Pax4 expression in Type 2 diabetic donors whereas long-term adiposity appears to favour suppression of the gene.

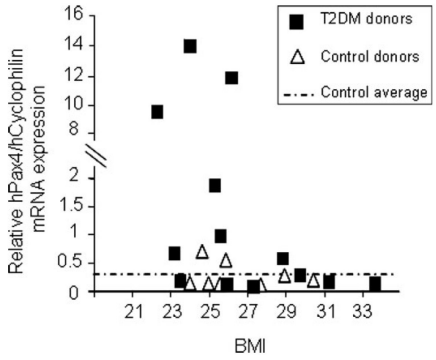

Figure 1. Pax4 mRNA expression levels are increased in human islets isolated from Type 2 diabetic donors with BMI between 22 and 26. Pax4 expression was measured by quantitative RT–PCR in isolated pancreatic islets from (■) T2DM (7 males and 6 females) and (Δ) non-diabetic control donors (6 males and 2 females). The average age of donors was 64 ± 10 years ($48 < \text{age} > 80$). Data are presented as relative mRNA abundance levels normalized to the housekeeping gene transcript cyclophilin levels in function of body mass index (BMI). Each dot represents an individual donor.

To determine whether development of Type 2 diabetes in donors with increased levels of Pax4 could be potentially correlated to polymorphisms and/or mutations associated with the disease (12–15,17), DNA isolated from seven donors (four diabetics and three non-diabetics) were sequenced in regions of interests in the *pax4* gene. Individual diabetes-linked polymorphisms or mutations could not be identified within the paired domain of the *pax4* gene (data not shown). Interestingly, three of the four Type 2 diabetic donors as well as two of the three non-diabetic donors carried a previously described single nucleotide polymorphism (SNP rs698406; G to C) at position 1298 (relative to the transcriptional initiation site). This SNP is located within intron 3 and has not yet been assigned any functional phenotype.

Glucose-induced insulin release stimulates *Pax4* gene transcription in human islets

To validate the hypothesis that glucose is responsible for the *in vivo* up-regulation of Pax4 expression, primary culture of human control islets were exposed to increasing concentrations of glucose for 24 and 48 h. A 3-fold increase in Pax4 mRNA levels was observed in islets treated with 25 mM glucose, whereas transcript levels returned to basal values at 33 mM glucose (Fig. 2A). No significant differences in the induction of Pax4 expression were observed between 24 and 48 h. Correspondingly, Ipf1 mRNA levels also exhibited a bell-shape expression pattern reaching maximal induction of approximately 2-fold at 25 mM glucose before decreasing to basal levels at 33 mM glucose. We have previously demonstrated that low concentrations of IL-1β via the FAS-FLIP signalling pathway induced Pax4 expression, whereas high concentrations inhibited mRNA levels of the transcription factor (20). Corroborating these studies, we found that

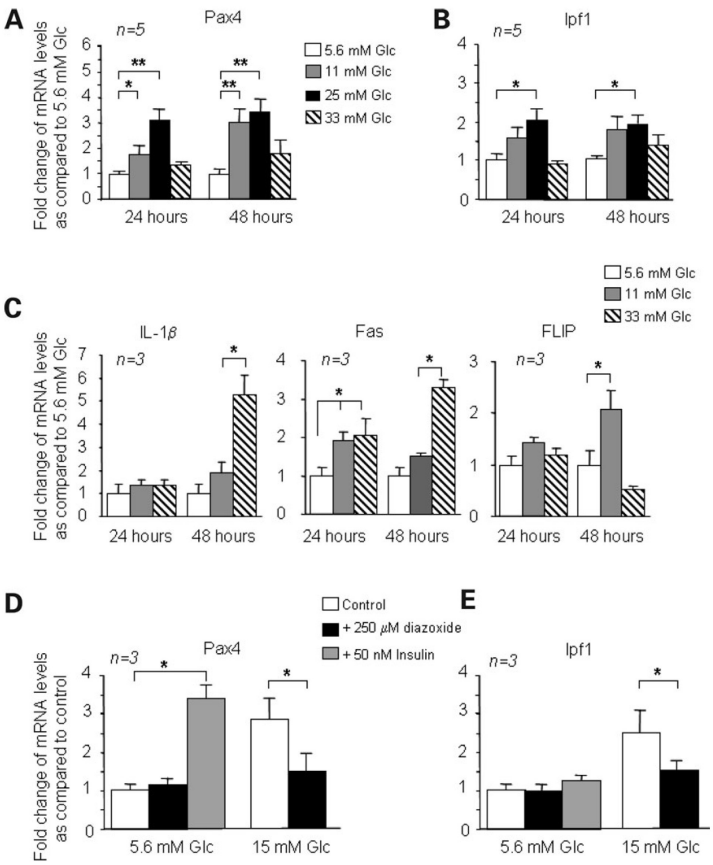

Figure 2. Glucose stimulates *Pax4* gene expression in isolated human islets. (**A**) Pax4, (**B**) Ipf1, (**C**) IL-1β, Fas and FLIP mRNA levels in islets treated with increasing doses of glucose as indicated in the figure legends. Quantitative RT–PCR using RNA purified from cultured human islets was performed on Pax4 and the mentioned genes. Data are presented as fold change of mRNA levels when compared with 5.6 mM Glc normalized to the cyclophilin transcript. Values represent the mean ± SEM of 3–5 independent experiments performed in duplicates. (**D** and **E**) Islets were incubated with 5.6 mM Glc in the absence or presence of insulin (50 nM) or with 15 mM Glc in the absence or presence of the non-selective K_{ATP} channel opener diazoxide (250 μM). (**D**) Pax4 and (**E**) Ipf1 transcripts abundance levels were estimated by quantitative RT–PCR. Statistical significance was tested by Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$.

glucose dose dependently induced IL-1β and FAS transcript levels reaching maximal induction of 5- and 3-fold, respectively, at 33 mM glucose whereas the expression pattern of the caspase-8 inhibitor FLIP mimicked that of Pax4 (Fig. 2C). These results suggest that islets exposed to elevated glucose concentrations such as in Type 2 diabetic patients will produce increasing amounts of IL1-β and FAS ultimately inhibiting *pax4* gene transcription and induce cell death (20).

As the predominant function of glucose metabolism in β-cells is to promote insulin secretion which may then have an autocrine effect on islet cells, we investigated whether insulin could stimulate Pax4 expression. Addition of exogenous insulin in the presence of 5.6 mM glucose prompt a 3.5-fold increase in Pax4 mRNA levels when compared with control islets whereas Ipf1 levels remained constant (Fig. 2D and E). Addition of diazoxide, a K_{ATP} channel opener which blocks nutrient-induced insulin secretion, completely abrogated the effect of 15 mM glucose on Pax4 as well as on Ipf1 expression (Fig. 2D and E). Taken together our data suggests that most likely insulin released in response to high glucose is the main stimulator of Pax4 transcription. In contrast, glucose and not insulin appears to be the main stimulator of Ipf1 transcription in human islets (22).

Pax4 expression in human islets is induced by activin A, betacellulin and GLP-1

In order to determine whether other growth factors stimulated *pax4* gene expression, human islets were cultured in the presence of either activin A (a member of the TGF-β family) or betacellulin (a member of the EGF family). Pax4 mRNA levels were increased by approximately 5-fold in islets treated with either 0.5 nM activin A or betacellulin for 24 or 48 h (Fig. 3A). Interestingly, the combination of activin A and betacellulin in the presence of either 5.6 or 15 mM glucose did not further increase Pax4 expression when

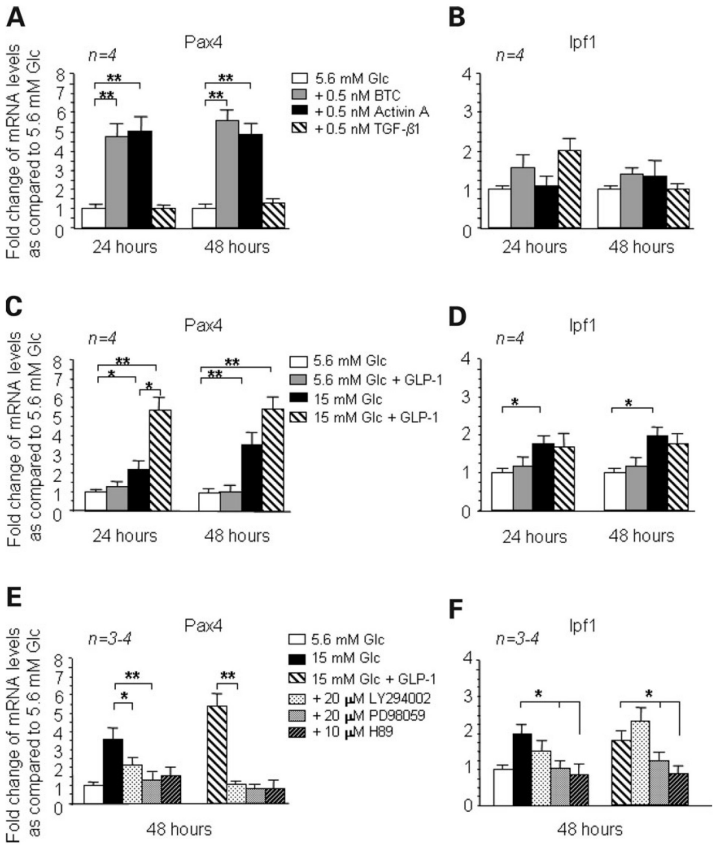

Figure 3. Activin A, betacellulin and the incretin GLP-1, in combination with glucose, increase Pax4 mRNA levels in human islets. (**A**) Pax4 and (**B**) Ipf1 mRNA levels in islets treated with activin A, betacellulin or TGF-ß1 as indicated in the figure legend. Quantitative RT–PCR analysis was performed as described in Figure 2. (**C**) Pax4 and (**D**) Ipf1 mRNA levels in islets incubated with glucose in the absence or presence of GLP-1 (10 nM), as indicated on the graph. (**E**) Pax4 and (F) Ipf1 mRNA levels in islets incubated with glucose, GLP-1, the PI3-kinase inhibitor LY294002, the MEK1/2 specific inhibitor PD98059 and the PKA-specific inhibitor H89, as indicated on the graph. The results are normalized to cyclophilin and are expressed as fold change of mRNA when compared with control. Data are expressed as the mean ± SEM of four independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

compared with individual mitogens (data not shown). As in rat islets (5), TGF-β1 had no consequence on Pax4 expression. Ipf1 mRNA levels were not significantly increased by activin A, betacellulin or TGF-β1 treatments (Fig. 3B). We next determine the impact of GLP-1, a new therapeutic agent for the treatment of diabetes which has been shown to increase β-cell mass in mouse and rat pancreas as well as promoting cell proliferation in INS-1 cells (23–27). GLP-1 (10 nM) in combination with 5.6 mM glucose had no stimulatory effect while in the presence of 15 mM glucose the incretin elicited a 6-fold increase in Pax4 mRNA levels at either 24 or 48 h (Fig. 3C). This increase was significantly greater than that of 15 mM glucose alone at 24 h indicating that GLP-1 potentiated the effect of the sugar on Pax4 stimulation. Similar results were obtained with the long acting analogue of GLP-1, exendin-4 (data not shown).

Investigation as to which particular secondary signal might evoke an increase in Pax4 expression in response to glucose alone or in combination with the incretin was then conducted. Inhibition of the PI3K pathway with LY294002 repressed both glucose and glucose/GLP-1-mediated increase in Pax4 expression (Fig. 3E). Likewise, Pax4 induction was completely blunted by the MEK1/2 specific inhibitor PD98059 which blocks the ERK1/2 axis of the insulin signalling pathway (Fig. 3E). To determine the contribution of the cAMP-PKA pathway in the stimulation of Pax4 expression, islets cultured in the presence of 15 mM glucose with or without of GLP-1 were treated with the PKA inhibitor H89. Induction of Pax4 expression was abrogated in the presence of H89 (Fig. 3E). Taken together, these results indicate that glucose most likely via insulin enhance Pax4 expression through activation of both the ERK1/2 and PI3K branches of the insulin signalling cascade as well as the cAMP-PKA pathway. As cross talk between these three pathways has previously been established (28), incapacitating any one cascade results in complete inhibition of Pax4. Complete inhibition of

the stimulatory effect of GLP-1 in combination with 15 mM glucose on Pax4 expression confirms the strict dependency of the incretin action on the presence of the sugar.

Surprisingly, glucose-mediated stimulation of Ipf1 expression was not further increased by the addition of GLP-1 (Fig. 3F). Furthermore, PD98059 and H89 but not LY294002 inhibited induction of Ipf1 expression by glucose indicating that, in contrast to Pax4, stimulation of Ipf1 is not dependent on the PI3K pathway.

Mitogens and GLP-1-mediated activation of endogenous Pax4 does not increase human β-cell proliferation

We have previously demonstrated that stimulation of *pax4* gene expression by activin A and betacellulin coincided with rat islet cell proliferation (5). In parallel, GLP-1 was previously shown to stimulate rodent and murine β-cell replication (29,30). Thus, to determine whether mitogens- and GLP-1-elicited increases in Pax4 expression levels correlated with induction of human islet cell replication, BrdU-incorporation was evaluated in islets treated with various growth factors. Consistent with a previous study, we found that the total number of islet cells undergoing proliferation under non-stimulatory conditions was ~0.5% (Fig. 4A) (31). Astonishingly, addition of activin A, betacellulin or GLP-1 failed to induce replication (Fig. 4A). Longer incubation time in the presence of BrdU and growth factors (alone or in combination) as well as culturing cells on various substrata (up to 6 days) did not improve yield of labelled cells (data not shown). In contrast, rat islets exposed to GLP-1 or activin A displayed a 2.5-fold increase in BrdU-labelling while betacellulin elicited a 3-fold increase when compared with control 5.6 mM treated islets (Fig. 4B).

To address the possibility that human islet β-cells are refractory to these physiological stimuli *in vitro*, we investigated whether nuclear translocation of the proliferation marker Id2 (32,33) occurred in activin A treated islets. As expected, under control conditions Id2 was predominantly localized to the cytoplasm of both human and rat islet cells, whereas addition of activin A prompted nuclear translocation of the protein (Fig. 4C and D). Interestingly, translocation was only observed in β-cells despite presence of Id2 in all cell types. These results suggest that human β-cells are responsive to mitogens such activin A resulting in Pax4 stimulation and Id2 nuclear translocation but fail to subsequently enter into the S-phase.

Human islet proliferation is not significantly induced by adenoviral-mediated overexpression of human Pax4

In order to investigate whether supra-physiological levels of human Pax4 could force entry of cells into the replication phase, human islets were infected with a doxycycline inducible adenoviral construct bearing the human Pax4 cDNA tagged with a myc epitope. The latter was essential to reveal Pax4 by immunocytochemistry as no reliable antibodies for the transcription factor are commercially available. A 14-fold increase in Pax4 transcript levels was estimated subsequent to doxycycline treatment corresponding to a 2-fold increment when compared with islets incubated with activin A or betacellulin (compare Fig. 5A to 3A). Consistent with our previous studies in rat islets (5), overexpression of Pax4 did not alter Ipf1 mRNA levels. Interestingly, a small but significant 1.6-fold increase in transcript levels of the Pax4-target gene, Bcl-xL was found in doxycycline treated islets whereas Id2 mRNA levels maintained basal values when compared with untreated islets (Fig. 5B). The latter results would tend to suggest that human Pax4, in contrast to the mouse variant (5), is less efficient in activating downstream target genes and thus to stimulate proliferation. Consistent with this hypothesis, immunocytochemistry revealed that although Pax4 was expressed in ~60% of cell nuclei after doxycycline treatment (Fig. 5C), no significant increase in BrdU labelling could be discerned when compared with control untreated islets (Fig. 5D and E). In contrast, human islets transduced with the mouse variant of Pax4 employing the same viral transduction system, displayed a 10-fold increase in islet cell replication (Fig. 5F). As an activator and/or repressor domain was previously identified at the carboxy-terminal end of Pax4 (34), we investigated whether activin A may be required to activate the transcription factor and increase proliferation in transduced islets. No further increase in BrdU labelling was discerned in these islets when compared with non-induced Ad-hPax4-myc-infected islets (Fig. 5E). Similar attempts to stimulate proliferation in the presence of betacellulin and GLP-1 also failed (data not shown).

We next evaluated the capacity of both the human and mouse Pax4 to promote cell replication in rat islets. Subsequent to infection and doxycycline treatment, 70% of islet cells expressed either recombinant protein (Fig. 6A). However, similarly to human islets, only mouse Pax4 was capable of stimulating proliferation suggesting potential post translational modifications of the human protein that modulates its activity.

Pax4 gene expression in human and rat islets is regulated by epigenetic modifications

Aberrant DNA demethylation in the *pax4* gene promoter was recently shown to induce expression of the transcription factor in lymphocytes and promote haematologic malignancies (35). Furthermore, a member of the *id* gene family, Id4 was also found to be regulated by epigenetic modifications (36). These findings suggest that Pax4 expression as well as downstream target genes may be epigenetically regulated in human islets thereby blocking activation of the replication program. Consistent with this premise, human islets treated with increasing concentrations of the DNA methyltransferase inhibitor, 5-Aza-2′-deoxycytidine (5′-AZA) for 72 h exhibited a gradual increase in Pax4 mRNA levels reaching maximal induction of 3-fold with 20 μM of the drug (Fig. 7A). In contrast, Ipf1 mRNA levels were unaltered. Nonetheless, neither Bcl-xL nor Id2 gene expression was increased in the presence of 5′-AZA (Fig. 7B). In contrast, a 10-fold stimulation in Pax4 transcript levels as well as a concomitant increase of 2.5- and 3-fold in Id2 and Bcl-xL gene expression was observed in rat islets incubated with 10 μM 5′-AZA (Fig. 7C and D). These results highlight a novel regulatory mechanism of *pax4* gene expression through epigenetic modification in rat and human islets. However, alleviation of this regulatory checkpoint was still not sufficient to activate downstream target genes. The latter conclusion combined with the adenoviral transduction studies showing a modest increase in only Bcl-xL transcript in the presence of supra physiological levels of human Pax4,

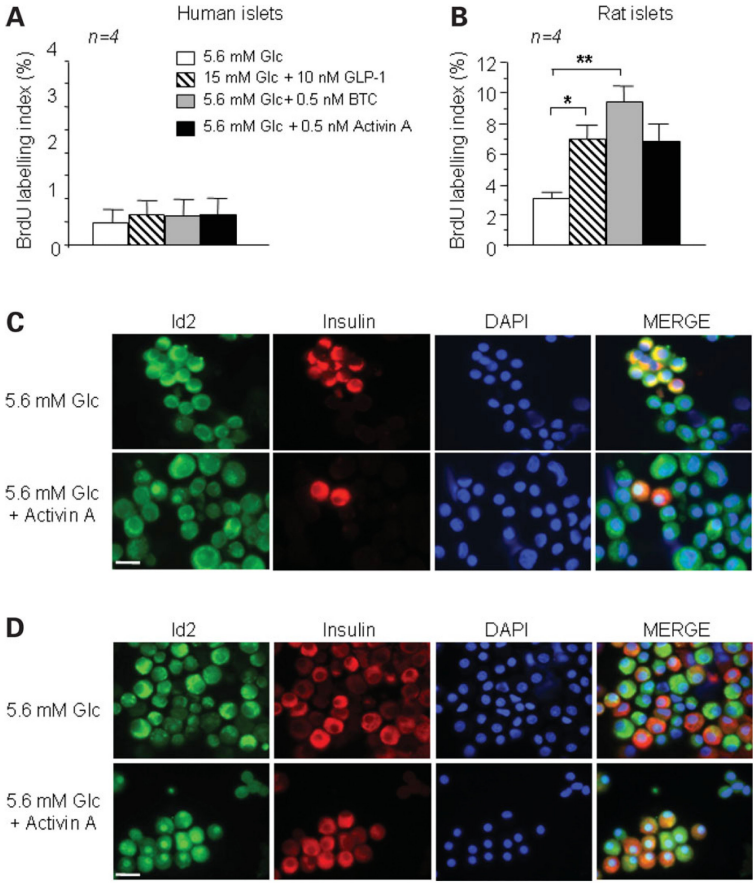

Figure 4. Activin A, betacellulin or GLP-1 do not induce human β-cell proliferation. Islet cell proliferation was measured by BrdU incorporation in (**A**) human and (**B**) rat islets treated with the indicated growth factors for 4 days. Dispersed islet-cells immunostained for BrdU were counted under a fluorescent microscope and results are depicted as a percentage of BrdU positive cells over the total amount of cells identified by DAPI staining. Data represent the mean ± SE of 4 independent experiments, each representing more than 1000 cells per condition. $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**–**D**) The downstream target of Pax4, Id2 is expressed in the cytoplasm of human and rat islets and translocates to the nucleus of β-cells in the presence of activin A (0.5 nM). Immunofluorescent detection of Id2 (green), insulin (red) as well as DAPI nuclei staining (blue) in dispersed human (**C**) and rat (**D**) islet cells incubated in the absence or presence of activin A (0.5 nM) for 48 h. The merge image of the Id2 and insulin is shown. Bars, 50 μm.

prompt us to investigate whether the human Pax4 protein when compared with the rat or mouse variant might be less efficient in *trans*-activating target genes. Electrophoretic mobility shift assays (EMSAs) using equal amounts of recombinant mouse and human Pax4 (Fig. 7F) revealed a much stronger binding of the mouse variant to the *glucagon* gene promoter element G3 (Fig. 7E) when compared with the human protein. The latter therefore substantiates the concept that the human protein is a poor *trans*-activator and requires much higher levels than its murine counterpart to stimulate transcription and most likely proliferation.

DISCUSSION

The beneficial effect of Pax4 on β-cell replication and survival has been well established in rodent islets. A similar positive outcome was also demonstrated in human islets in which murine Pax4 was overexpressed using adenovirus (5). However, regulation of endogenous *pax4* gene as well as its functional role in human islet plasticity under physiological or pathophysiological conditions remains obscure. Herein we show that islets derived from Type 2 diabetic donors with BMI between 22 and 26 have elevated levels of Pax4 transcript when compared with non-diabetic controls whereas expression levels are indistinguishable between the two groups with BMI greater than 26. Previous work reported a slight increase in Pax4 expression in Type 2 diabetes islets relative to controls (37). The relatively stable expression level of Pax4 in non-diabetic patients across BMI values suggests that hyperglycaemia acts as an important inducer of Pax4 expression and potentially cell replication in diabetic islets. Consistent with this premise, Pax4 expression was

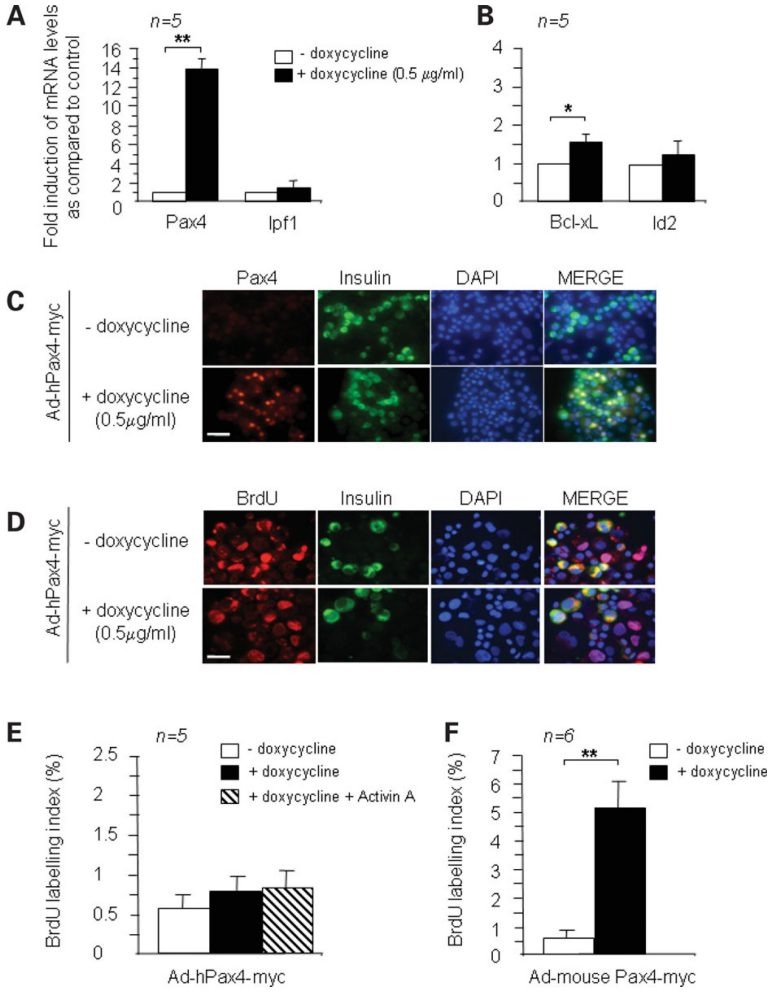

Figure 5. Adenoviral-mediated overexpression of human Pax4 does not stimulate replication in human islets. Islets were co-infected with Ad-hPax4-myc or Ad-mousePax4-myc along with Ad-X Tet-On as described in Materials and Methods. Doxycycline-dependent activation of human PAX4 was then assessed 48 h later by (**A**) quantitative RT–PCR and (**C**) immunohistochemistry; myc epitope (red), insulin (green) and DAPI (blue). Pax4 was detected via the myc epitope in the nuclei of ~60% of human islet cells cultured in the presence of doxycycline (0.5 μg/ml), while no basal induction of Pax4 was observed in the absence of doxycycline. Bars, 50 μm. (**B**) Bcl-xL and Id2 transcript levels in Ad-hPax4-myc transduced islets incubated with or without doxycycline. Each value represents mean ± SEM of 5 independent experiments. Islet cell proliferation was measured by BrdU incorporation in human islets infected with (**D** and **E**) Ad-hPax4-myc or with (**F**) Ad-mousePax4-myc and cultured with (0.5 μg/ml) or without doxycycline and activin A (0.5 nM) in the presence of BrdU (10 μM) for 4 days. (D) Islets were immunostained for BrdU (red), insulin (green) and DAPI (blue). Bars, 50 μm. (E–F) Dispersed islet cells immunostained for BrdU were counted under a fluorescent microscope and results are depicted as a percentage of BrdU positive cells over the total amount of cells as determined by DAPI staining. Data show the mean ± SE of 5–6 independent experiments, each representing more than 1000 cells per condition. $^{*}P < 0.05$; $^{**}P < 0.01$.

stimulated in human islets cultured in either 11 or 25 mM glucose while transcript levels returned to basal levels with higher concentrations. A similar bell-shaped Pax4 expression pattern was reported in human islets cultured with increasing concentrations of Il-1β. Indeed, low concentrations of the cytokine correlated with enhanced Pax4 expression and improved islet function whereas high levels inhibited Pax4 and induced apoptosis (20). In the current study, we show that Il-1β and its downstream target FAS were dose-dependently increased by glucose. It is therefore tempting to speculate that initial increases in Pax4 expression detected in human diabetic islets could be partly conveyed by glucose-induced Il-1β generation and release, whereas chronic exposure to the cytokine becomes inhibitory and detrimental to cells. The latter would also corroborate with the observation that Type 2 diabetic islets expressed the cytokine as a result of hyperglycaemia (38). We also demonstrate that insulin released in response to high glucose plays a pivotal

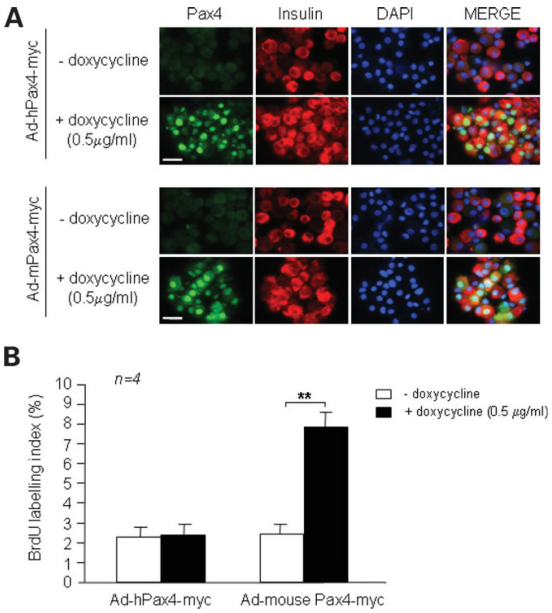

Figure 6. Adenoviral-mediated overexpression of human Pax4 does not stimulate replication in rat islets. Rat islets were co-infected with either Ad-hPax4-myc or Ad-mousePax4-myc and Ad-X Tet-On as described in Materials and Methods. Doxycycline-dependent activation of PAX4 was then assessed 48 h later by (**A**) immunohistochemistry; myc epitope (green), insulin (red) and DAPI (blue). Bars, 50 μm. (**B**) Islet cell proliferation was measured by BrdU incorporation in rat islets infected with Ad-hPax4-myc or with Ad-mousePax4-myc and cultured with (0.5 μg/ml) or without doxycycline in the presence of BrdU (10 μM) for 4 days. Dispersed islet cells immunostained for BrdU were counted and results are depicted as a percentage of BrdU positive cells over the total amount of cells. Data show the mean ± SE of four independent experiments, each representing more than 800 cells per condition. $^{**}P < 0.01$.

role in stimulating Pax4 expression. The latter correlates with recent findings showing that exogenous insulin protected human islets from apoptosis induced by serum withdrawal (22). Interestingly, islets of Type 2 diabetic donors with BMI values greater than 26 exhibited Pax4 mRNA levels identical to those of non-diabetic islets suggesting that adiposity may repress hyperglycaemia-induced Pax4 expression. Of note, palmitate known to be elevated in plasma of obese individuals, has been reported to attenuate human β-cell proliferation (39). Moreover, prolonged exposure to free fatty acids induced β-cell apoptosis in human islets (40). The current data thus suggest a causal association between the expression pattern of endogenous Pax4 and dynamic alterations observed in β-cell mass in response to hyperglycaemia.

Similar to rat islets, activin A and betacellulin increased Pax4 expression in human islets (5). However, this stimulation was approximately 2-fold lower in magnitude to that observed in rat islets. Interestingly, an analogous diminished response to glucose was also apparent comparing human and rat islets (this study and reviewed in 41). Taken together, human islets appear more resistant to growth factors. The latter does not emerge from an *in vitro* artefact or an effect of donor factors as human islets were shown to have improved function *in vivo* subsequent to prolonged culture time (42,43). However, we discovered that Pax4 expression was regulated by epigenetic modification which may impose restriction on the level of transcriptional activation by various stimuli in human islets. It will be of interest to determine whether combined treatment of islets with mitogens and 5′-AZA induces Pax4 expression to levels detected in rat islets. Addition of GLP-1 to 15 mM glucose further increased Pax4 mRNA levels indicating that the incretin potentiates the effect of glucose. This induction was found to be dependent on cAMP/PKA, ERK1/2 and PI3K activities suggesting that extensive cross talk between the G-protein coupled receptor and tyrosine coupled receptor transduction pathways is taking place before converging onto Pax4. Consistent with the premise that the effect of GLP-1 is glucose dependent, all three inhibitors completely abrogated the glucose-mediated stimulation of Pax4. Friedrichsen *et al*. (30) have recently demonstrated that GLP-1 induced *cyclinD1* gene transcription in rat islets with the subsequent induction of β-cell replication. Interestingly, the latter effects were completely blocked by the inhibitors LY294002, PD98050 and H89. In an independent study, adenoviral-overexpression of cyclin D1 in human islets caused a 2.5-fold increase in thymidine incorporation (31). Thus, it will be of interest to determine whether induction of cyclin D1 and proliferation by glucose and the incretin is conveyed by Pax4.

Our data demonstrate that induction of Ipf1 expression was refractory to activin A, betacellulin as well as TGF-β1. To our knowledge, no studies have investigated the impact of these mitogens on the regulation of Ipf1 in mature human islets. However, conditional expression of Smad7 in mouse pancreatic islets which disrupted the TGF-β signalling pathway had no consequence on Pdx1 (mouse homologue of Ipf1) mRNA levels (44) indicating that expression of the transcription factor is not regulated by TGF signalling in either murine or humans. Intriguingly, GLP-1 did not further stimulate Ipf1 expression induced by high glucose concentrations. This is sharp contrast to a single study performed with human foetal pancreas showing that Exendin-4 (a long-acting derivative of GLP-1) up-regulated expression of Ipf1 and accelerated differentiation and maturation of β-cells from precursor cells (45). This apparent discrepancy may be reconciled by the premise that Ipf1 expression during development may be regulated by GLP-1 whereas in mature human islet *ipf1* gene transcription is refractory to the incretin. The irrefutable beneficial impact of GLP-1 on β-cell function in human subjects could still involve post-translational regulation of Ipf-1. In support of this hypothesis, insulin was recently found to stimulate Ipf1 nuclear translocation in human β-cells correlating with decreased apoptosis (22).

An unexpected finding of the current study was the complete absence of increased human islet proliferation by activin A, betacellulin, glucose or GLP-1. Astonishingly, very few studies have successfully demonstrated growth factor-induced proliferation of adult human β-cells (reviewed in 28) while the impact of GLP-1 on human islet mass expansion remains to be established. A recent study demonstrated that age of donor correlated with decreased proliferative capacity of human β-cells which could account for our inability to stimulate proliferation (46). However, stimulation

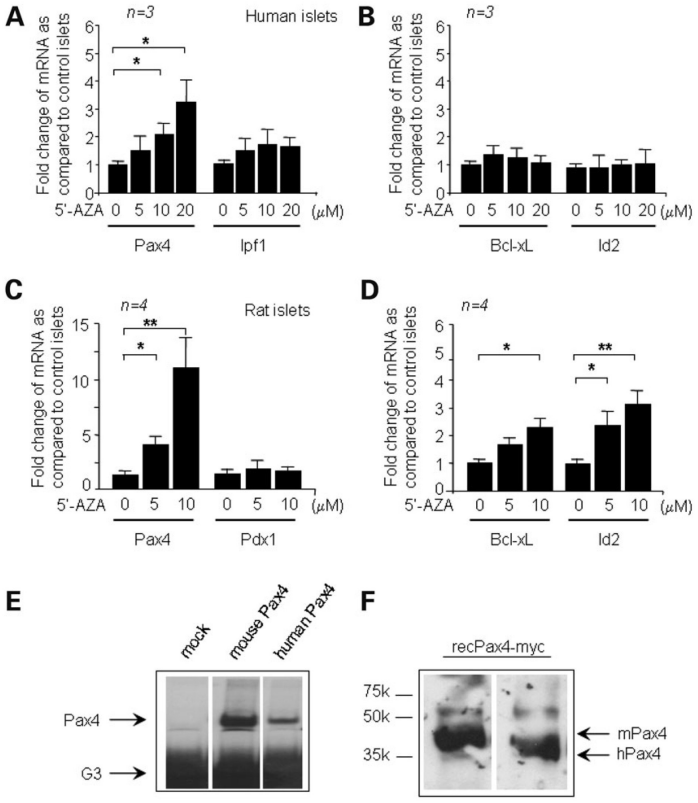

Figure 7. Inhibition of DNA methylase induces *Pax4* gene expression in human and rat islets. Isolated (**A** and **B**) human and (**C** and **D**) rat islets were treated with increasing concentrations of the DNA methyltransferase inhibitor, 5′-Aza-2′-deoxycytydine (5′-AZA) for 72 h. Pax4, Ipf1/Pdx1, Bcl-xL and Id2 transcripts abundance levels were estimated by quantitative RT–PCR. Data are presented as fold change of mRNA levels when compared with control islets normalized to cyclophilin and represent the mean ± SEM of at least three independent experiments performed in duplicates.*$P < 0.05$; **$P < 0.01$. (**E**) EMSA using the radiolabelled G3 element of the glucagon gene promoter and the recombinant proteins mouse and human Pax4-myc. An equal amount of protein was applied in each lane (Fig. 6F). Mouse Pax4 bound strongly to the G3 element whereas the binding of the human Pax4 was less efficient. (**F**) Western blotting of the recombinant proteins mouse and human Pax4-myc using an anti myc epitope antibody. The same anti-myc serum was used for Western blotting and immunofluorescence.

of Pax4 expression and the concomitant nuclear translocation of the proliferative marker Id2 by mitogens indicated that growth factor-mediated signalling was functionally adequate to set in motion the replication programme but that downstream activation of target genes was abortive and/or blocked. Overexpression of human Pax4 confirmed this assumption as Id2 expression was not induced while Bcl-xL transcript levels were slightly increased in transduced human islets. The failure of human Pax4 to stimulate downstream target genes did not appear to stem from epigenetic modification, as treatment with 5′-AZA fail to induce expression of either Bcl-xL or Id2 while Pax4 transcription was activated. Interestingly, Pax4 as well as Id2 and Bcl-xL were strongly induced subsequent to 5′-AZA treatment of rat islets suggesting that human Pax4 is less efficient in *trans*-activating its downstream target genes. This premise was confirmed by EMSA studies and substantiated a previous study showing weak interaction of the human Pax4 protein with its cognate DNA binding sequence when compared with the mouse protein (14). The latter results thus reconcile the observation that overexpression of mouse but not human Pax4 in either human or rat islets was capable of inducing cell replication. The question arises as why rodent Pax4 is a more robust *trans*-activator when compared with its human counterpart. Alignment of the three proteins revealed an 89% similarity in the DNA binding domains whereas a 50% amino acid divergence was estimated in the carboxy-terminal end (amino acids 230–352) of the rat and mouse sequence to that of the human. This segment of the transcription factor was previously shown to contain a repressor/*trans*-activator domain (34,47). Interestingly, human insulinomas were shown to contain high levels of a Pax4 variant lacking the carboxy-terminal end of the protein (48). It is therefore tempting to speculate that a nuclear co-factor interacts with the carboxy-terminal end of the human protein regulating DNA binding. This possibility is currently being explored with the use of chimeras containing the amino-terminal end of the human Pax4 linked to the carboxy-terminal end of the mouse protein and the reverse.

Alternatively, phosphorylation of serine/threonine residues in this region may also regulate binding activity has previously demonstrated for Pdx1 and NeuroD (49,50).

In summary, this study demonstrates that Pax4 expression is increased in Type 2 diabetic donor islets, an effect which is mediated by high circulating blood glucose and inhibited by increased adiposity. The latter is consistent with the hypothesis that human islet β-cell mass initially expands to compensate for insulin resistance but that there is a long-term failure and development of Type 2 diabetes (51). Consistent with these *in vivo* findings, we show that Pax4 expression is stimulated in human islets cultured in the presence of mitogens, glucose, insulin and GLP-1. However, due to potential functional divergence in the human and murine Pax4 protein, β-cell replication was not induced under any experimental conditions. The latter highlights the fundamental differences between human and murine/rodent islet physiology and emphasizes the importance of validating results obtained with animal models in human tissues. This dichotomy is further reinforced by recent findings showing that cdk-4 which is essential for murine β-cell replication in totally absent in human islets (52). Thus, elucidating the mechanism by which the activity of the human Pax4 is restrained should facilitate the development of a regenerative therapy for the treatment of diabetes.

MATERIALS AND METHODS

Cell culture

Pancreatic islets were isolated from 7-week-old male Wistar rats (Elevage Janvier, Le Genest-St-Isle, France) by collagenase digestion (53), handpicked and cultured for 24 h in 11.5 mM glucose/RPMI-1640 supplemented with, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin (Sigma-Aldrich, Basel, Switzerland). Freshly isolated human islets were obtained from either the Cell Isolation and Transplantation Laboratory in Geneva or from Ulm University in Ulm and maintained in CMRL-1066 (at 5.6 mM glucose) supplemented with 10% FCS, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin for 24 h. Subsequently, human islets were cultured for 24 and 48 h in the presence of increasing concentrations of glucose (5.6, 11, 25 and 33 mM) with or without 10 nM GLP-1 or 250 μM diazoxide. In some instances, 20 μM LY294002 (PI3-kinase inhibitor), 20 μM PD98050 (MAPKK inhibitor) or 10 μM H89 (PKA inhibitor) were individually added to the culture media. Islets were also exposed to 50 nM insulin, 0.5 nM betacellulin (BTC), activin A, TGF-β1 or increasing concentrations of 5′-Aza-2′-deoxycytidine. All chemicals were purchased from Sigma-Aldrich.

Additionally, islets from Type 2 diabetic donors were isolated at the Metabolic Unit at the University of Pisa as previously described (54) and processed for RNA extraction (see below).

Adenoviral constructions

The human full length Pax4 cDNA was amplified from human islet-derived RNA and initially cloned into the pcDNA3.1/myc-His expression vector (Invitrogen, Basel, Switzerland). Subsequently, the Pax4-myc DNA fragment was subcloned into the pTRE-Shuttle2 vector (Takara Bio Europe, St-Germain-en-Laye, France). The inducible cassette was then transferred into the Adeno-X viral DNA to generate the recombinant adenovirus Ad-hPax4-myc. The mouse Pax4 cDNA viral construct, Ad-mPax4-myc, was previously described in (5).

Adenoviral infection of islets

Human or rat islets were co-infected with either Ad-hPax4-myc or Ad-mPax4-myc along with the adenoviral construct harbouring the tetracycline transcriptional activator (Ad-X Tet-On) at a ratio of 2:1 (3.6×10^7 *pfu*/ml total viral particles). Islets were rinsed 90 minutes post infection and cultured in fresh media with or without 0.5 μg doxycycline.

Quantitative real time-PCR (QT-RT-PCR)

Total RNA from 50 islets was extracted using the Trizol reagent (Invitrogen) and 2 μg were converted into cDNA as previously described (55). Primers for cyclophilin, Id2, Bcl-xL, IL-1β, Fas, FLIP, iNOS and Pax4 were designed using the Primer Express Software (Applera Europe, Rotkreuz, Switzerland) and sequences can be obtained on the Web page of the corresponding author (http://phym.unige.ch/groupes/gauthier/index.php). QT-RT-PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe) and PCR products were quantified using the SYBR Green Core Reagent kit (53). Two distinct amplifications derived from at least 3 independent experiments were performed in duplicate for each transcript and mean values were normalized to the mean value of the reference mRNA cyclophilin. Authenticity of each amplicon was verified by DNA sequencing.

Immunohistochemistry

Subsequent to treatment, islet single cell suspensions were obtained using trypsin and concentrated on glass cover slips by cytocentrifugation. Cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Mouse and human recombinant Pax4 myc tagged proteins were visualized by immunohistochemistry using an antibody against the myc epitope (dilution 1:200; Invitrogen). Immunochemical detection of Id2 was performed using a rabbit anti-human polyclonal antibody (dilution 1:200, Santa Cruz, USA) while insulin immunostaining was performed as previously described (56). Nuclei were then stained with DAPI (10 μg/ml; Sigma). Cover slips were mounted using DAKO fluorescent mounting medium and visualized using a Zeiss Axiophot I.

Cell proliferation

Islets cultured in standard media containing 10% FCS and supplemented with growth factors were labelled with 10 μM BrdU for up to 6 days. Proliferation was estimated using an immunohistochemical assay kit as described by the manufacturer (BrdU labelling and detection Kit, Roche Diagnostics, Switzerland).

Results are expressed as the percentage of BrdU-positive cells over the total amount of islet cells identified by nuclear DAPI staining and are depicted as a BrdU labelling index.

Recombinant Pax4 preparation and electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as previously described (57) using an oligonucleotide corresponding to the rat *glucagon* gene promoter element G3 (58) along with either human or mouse recombinant Pax4 protein generated from an *in vitro* transcription and translation system (Promega Inc., Wallisellen, Switzerland).

Western blotting

In vitro produced human and mouse recombinant Pax4 proteins were resolved on a 10% SDS–polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% milk powder and then incubated with a myc-epitope antibody (Invitrogen). Immunoreactive products were revealed by enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA) using horseradish peroxidase coupled secondary antibodies.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Where indicated, the statistical significance of the differences between groups was estimated by Student's unpaired *t*-test. * and ** indicate statistical significance with $P < 0.05$ and $P < 0.01$, respectively.

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Conflict of Interest statement. None to declare.

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7.1.3 TCF7L2 Regulates β-Cell Survival and Function in Human Pancreatic Islets.

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ORIGINAL ARTICLE

Transcription Factor 7-Like 2 Regulates β-Cell Survival and Function in Human Pancreatic Islets

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OBJECTIVE—Type 2 diabetes is characterized by impaired insulin secretion in response to increased metabolic demand. This defect in β-cell compensation seems to result from the interplay between environmental factors and genetic predisposition. Genome-wide association studies reveal that common variants in transcription factor 7-like 2 (TCF7L2) are associated with increased risk of type 2 diabetes. The aim of the present study was to establish whether TCF7L2 plays a role in β-cell function and/or survival.

RESEARCH DESIGN AND METHODS—To investigate the effects of TCFL7L2 depletion, isolated islets were exposed to TCF7L2 small interfering RNA (siRNA) versus scrambled siRNA, and β-cell survival and function were examined. For TCF7L2 overexpression, islets were cultured in glucose concentrations of 5.5–33.3 mmol/l and the cytokine mix interleukin-1β/γ-interferon with or without overexpression of TCF7L2. Subsequently, glucose-stimulated insulin secretion (GSIS), β-cell apoptosis [by transferase-mediated dUTP nick-end labeling assay and Western blotting for poly(ADP-ribose) polymerase and Caspase-3 cleavage], and β-cell proliferation (by Ki67 immunostaining) were analyzed.

RESULTS—Depleting TCF7L2 by siRNA resulted in a 5.1-fold increase in β-cell apoptosis, 2.2-fold decrease in β-cell proliferation ($P < 0.001$), and 2.6-fold decrease in GSIS ($P < 0.01$) in human islets. Similarly, loss of TCF7L2 resulted in impaired β-cell function in mouse islets. In contrast, overexpression of TCF7L2 protected islets from glucose and cytokine-induced apoptosis and impaired function.

CONCLUSIONS—TCF7L2 is required for maintaining GSIS and β-cell survival. Changes in the level of active TCF7L2 in β-cells from carriers of at-risk allele may be the reason for defective insulin secretion and progression of type 2 diabetes. ***Diabetes* 57:645–653, 2008**

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DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; GLP-1, glucagon-like peptide 1; GSIS, glucose-stimulated insulin secretion; IFN-γ, γ-interferon; IL, interleukin; KRBB, Krebs-Ringer bicarbonate buffer; PARP, poly(ADP-ribose) polymerase; siRNA, small interfering RNA; siScr, scrambled control siRNA; siTCF7L2, islets depleted for TCF7L2 by siRNA to TCF7L2; SNP, single nucleotide polymorphism; TCF7L2, transcription factor 7-like 2; TUNEL, transferase-mediated dUTP nick-end labeling.

Type 2 diabetes is characterized by impaired insulin secretion and insulin resistance (1). Type 2 diabetes manifests when insulin secretion fails to adaptively increase to increased insulin demand. This might be due to defective β-cell mass and/or impaired β-cell function (2–3). The underlying mechanisms of β-cell failure in type 2 diabetes are still unknown, but recent genome-wide association studies have offered some new targets of interest.

Grant et al. (4) reported linkage between a variant of the transcription factor 7-like 2 (TCF7L2) and type 2 diabetes in Danish and U.S. cohorts (4). This finding has been confirmed by several other genome-wide studies (4–13) and in numerous populations (13). TCF7L2 (previously known as TCF-4) is an important downstream target of the canonical WNT signaling pathway (14). It is highly expressed in most human tissues, including heart, placenta, lung, brain, liver, adipose tissue, kidney, and pancreatic β-cells, but not in the skeletal muscle (10). Interestingly, TCF7L2 expression in adipose tissue is decreased in obese subjects with type 2 diabetes (10).

TCF7L2 has been implicated in glucose homeostasis through the regulation of pro-glucagon gene expression, which encodes glucagon-like peptide 1 (GLP-1) in intestinal cells (15).

The single nucleotide polymorphisms (SNPs) in the TCF7L2 gene that show linkage with type 2 diabetes are all found in the noncoding regions (13). It is unknown whether changes in TCF7L2 expression levels in pancreatic islets directly influence β-cell function or survival. Because type 2 diabetes is characterized by impaired β-cell function and increased β-cell apoptosis, we sought to establish whether islet expression levels of TCF7L2 impact glucose-induced insulin secretion and vulnerability to apoptosis. After manipulating levels of TCF7L2, we examined glucose-induced insulin secretion by perifusion and β-cell survival after exposure to pro-apoptotic glucose concentrations and cytokines.

RESEARCH DESIGN AND METHODS

Islet isolation and culture. Human islets were isolated from pancreata of five healthy organ donors at the University of Illinois at Chicago as described previously (16) and shipped to UCLA directly after isolation. Islet purity of these five preparations was >95%, as judged by dithizone staining. Human islets were cultured in CMRL-1066 medium containing 5.5 mmol/l glucose and 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Invitrogen, Carlsbad, CA). Mouse islets were isolated using Collagenase type 4 (Worthington, Lakewood, NJ) as described previously (17) and cultured in RPMI 1640 containing 11.1 mmol/l glucose. In culture, a glucose concentration of 11.1 mmol/l accomplishes the lowest frequency of β-cell apoptosis in mouse islets (18–20). Human and mouse islets were precultured for 24 h before the experiment and then plated on matrix-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel) (21). For the perifusion

experiment, islets were maintained in suspension. For experimental treatment of islets, medium was changed to culture medium containing 5.5, 11.1, or 33.3 mmol/l or 5.5 mmol/l plus 2 ng/ml recombinant human interleukin (IL)-1β plus 1,000 units/ml recombinant γ-interferon (IFN-γ) (R&D Systems, Minneapolis, MN) for 96 h.

RNA interference and plasmid transfection. Small interfering RNA (siRNA)-Lipofectamine2000 complexes and DNA-Lipofectamine2000 complexes were prepared according to the manufacturer's instructions (Lipofectamine2000; Invitrogen) using 50 nmol/l siRNA to TCF7L2 (RNAs of 21 nucleotides, designed to target human TCF7L2; Stealth Select RNAi; Invitrogen) and scramble siRNA (Ambion, Austin, TX) or 3 μg/ml DNA of pCMV-TCF7L2 (full-length TCF7L2, from the Full-Length Mammalian Gene Collection; Invitrogen) or an pCMV-empty control plasmid. Islets were precultured for 24 h, medium was changed to OptiMEM (Invitrogen), and siRNA-Lipofectamine2000 complexes or DNA-Lipofectamine2000 was added. After an 8-h incubation, the transfection medium was aspirated and replaced by fresh culture medium with or without elevated glucose or cytokines for additional 4 days. To monitor transfection efficiency of siRNA into the islets, we transfected fluorescein-labeled nontargeted siRNA (Cell Signaling, Beverly, MA) and analyzed transfection under the fluorescent microscope during for 4 days of culture.

β-Cell apoptosis, replication, and TCF7L2 expression. For each independent experiment, 20 islets were plated in a 3-cm culture dish and exposed to the treatment conditions as indicated above. Four dishes per treatment group were used. After washing with PBS, islets were fixed with 4% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. β-Cell apoptosis was analyzed by the transferase-mediated dUTP nick-end labeling (TUNEL) technique (In Situ Cell Death Detection kit, AP; Roche Diagnostics, Indianapolis, IN). For β-cell proliferation, an anti-human Ki67 antibody was used (Zymed, San Francisco, CA), followed by detection using cy3-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). For β-cell TCF7L2 expression, islets were incubated with anti-TCF7L2 antibody (Cell Signaling, Beverly, MA), followed by detection using cy3-conjugated donkey anti-rabbit antibody (Jackson). In all experiments, islets were double/triple stained with guinea pig anti-insulin antibody (Dako, Carpinteria, CA), followed by detection using fluorescein-conjugated donkey anti-guinea pig antibody (Jackson). Islets were embedded in glycerol gelatin (Sigma) or Vectashield mounting medium (Vector Laboratories, Burlingame, CA), which visualized all cells by 4,6-diamidino-2-phenylindole (DAPI) staining. Fluorescence was analyzed using a Leica DM6000 microscope, and images were acquired using Openlab software.

Western blot analysis. For Western blot analyses, 100 islets/dish were plated in duplicates for each independent experiment. At the end of the incubation, islets were washed in PBS and lysed as described previously (22). Polyvinylidine fluoride filters were incubated with rabbit anti-TCF7L2 (2566), mouse anti-cleaved poly(ADP-ribose) polymerase (PARP) (9548), rabbit anti-caspase-3 (9961), rabbit anti-actin (4967), anti *p*-AKT (Serin473 9271) (all from Cell Signaling), followed by incubation with horseradish peroxidase–linked IgG peroxidase. The emitted light was captured on X-ray film after adding Immun-Star HRP Substrate (Bio-Rad Laboratories, Hercules, CA). Density of the bands was analyzed using Labworks 4.5 software (BioImaging Systems, Upland, CA).

RNA extraction and RT-PCR. Total RNA was isolated from cultured islets (100 islets/dish) as described previously (23). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche Diagnostics) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). Primers used were 5′-CTACCTAGTGTGCGGGGAAC-3′ and 5′-GCTGGTAGAGGGAG CAGATG-3′ (insulin), 5′-CTGGATTGGCGTTGTTTGTG-3′ and 5′-CTACAG CACTCCACCTTGGGA-3′ (PDX-1), and 5′-GAAGGAGCGACAGCTTCATA-3′ and 5′-GGGGGAGGCGAATCTAGTAA-3′ (TCF7L2) and compared with the housekeeping gene, 5′-AGAGTCGCGCTGTAAGAAGC-3′ and 5′-TGGTCTTGT CACTTGGCATC-3′ (α-Tubulin) and 5′-TCACCCACACTGTGCCCATCTACGA-3′ and 5′-CAGCGGAACCGCTCATTGCCA ATGG-3′ (β-actin).

Glucose-stimulated insulin secretion, static incubation. For each independent experiment, 20 islets were plated and exposed to the treatment conditions as indicated above. Four dishes per treatment group were used. For acute insulin release, islets were washed and preincubated (30 min) in Krebs-Ringer bicarbonate buffer (KRBB) containing 2.8 mmol/l glucose. The KRBB was then replaced by KRBB containing 2.8 mmol/l glucose for 1 h (basal), followed by an additional 1-h incubation in KRBB containing 16.7 mmol/l glucose. Islets were lysed in lysis buffer, and whole islet protein amount was measured by BCA protein assay (Pierce, Rockford, IL). Insulin was determined using a human insulin ELISA kit (Dako) or a mouse insulin ELISA kit (Alpco, Windham, NH).

Islet perifusion. Human islets plated in suspension dishes (100 islets/dish in triplicates) were precultured for 4 days with scrambled or TCF7L2 siRNA. Twenty islets of similar size from each treatment group were hand-picked and suspended in Bio-Gel P-2 beads (Bio-Rad) and placed in perifusion chambers as described previously (24). The perifusion system (ACUSYST-S; Cellex Biosciences, Minneapolis, MN) consisted of a multichannel peristaltic pump that delivered perifusate through six parallel tubing sets via a heat exchanger and six perfusion chambers at a constant rate of 0.3 ml/min. The perifusion buffers (KRBB) were preheated to 37°C, oxygenized with 95% O_2 and 5% CO_2, and delivered to the perifusion chambers containing the human islets. Islets were perifused for 1 h with KRBB containing 2.8 mmol/l glucose (perifusate was collected during the last 30 min every 5 min) for 40 min with KRBB containing 16.7 mmol/l glucose, for 30 min with KRBB containing 16.7 mmol/l glucose plus 100 nmol/l GLP-1 (fragment 7-37, human; Sigma), for 30 min with KRBB containing 2.8 mmol/l glucose, and for 10 min with 20 mmol/l KCl. The effluent was collected in 5-min (for basal and stimulated insulin secretion) and 2-min intervals (for KCl-induced insulin secretion) for determination of insulin concentrations. At the end of the perifusion, islet were collected by hand-picking and extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Insulin was determined using a human insulin ELISA kit (Alpco).

Statistical analysis. Immunostainings were evaluated in a randomized manner by a single investigator (L.S.) who was blinded to the treatment conditions. Data are presented as means ± SE and were analyzed by paired Student's *t* test or by ANOVA with a Bonferroni correction for multiple group comparisons.

RESULTS

Depletion in TCF7L2 results in impaired β-cell function. We investigated localization of TCF7L2 in human islets and found its expression in human β-cells, in confirmation with two previous reports (10,12). Triple-staining for TCF7L2, insulin, and DAPI revealed that TCF7L2 is expressed in the nucleus of β-cells (Fig. 1*A*, *1*). Plasmid overexpression of TCF7L2 resulted in increased TCF7L2 expression (Fig. 1*A*, *2*), whereas in islets depleted for TCF7L2 by siRNA to TCF7L2 (siTCF7L2), TCF7L2 was almost undetectable by immunohistochemistry (Fig. 1*A*, *3*).

To establish the effects of TCF7L2 on glucose mediated insulin secretion, we depleted TCF7L2 in pancreatic islets by exposure of mouse islets to three different siRNA sequences to TCF7L2 (siTCF7L2-1, -2, and -3) and human islets to one siRNA (siTCF7L2-2) (or scrambled control siRNA [siScr]) for 4 days. We achieved an average downregulation of TCF7L2 mRNA expression by 58 ± 10% (Fig. 1*F*) and 57 ± 12% protein expression in human (Fig. 3*E*) and in mouse islets (not shown). For evaluating transfection efficiency, islets were transfected with a fluorescein isothiocyanate (FITC)-labeled nontargeted siRNA and green fluorescence monitored over the 4-day culture period. Transfection efficiency was about 5% after 24 h and 75% after 48 h, which was maintained during the 4-day experiment (Fig. 1*A*, *4* and *5*).

We first tested the consequence of such downregulation on β-cell function in mouse islets. After a 4-day culture period with siRNA, we performed static incubation measurement of insulin secretion in mouse and human islets. No significant changes in insulin secretion were observed at basal levels at 2.8 mmol/l glucose. Also, a scrambled control sequence did not change basal or stimulated insulin secretion compared with untreated islets. In contrast, glucose-stimulated insulin secretion (GSIS) (16.7 mmol/l glucose) was decreased in mouse islets (1.6-, 2.3-, and 1.5-fold by siTCF7L2-1, -2, and -3, respectively, $P < 0.05$; Fig. 1*B*) compared with the scrambled control. This resulted in a 2.6-, 3.6-, and 2.6-fold decrease in insulin stimulatory index by siTCF7L2-1, -2, and -3, respectively (Fig. 1*C*). The same analysis was then performed in human islets. Because three different siRNA sequences showed the same results in mice, we performed the experiments in human

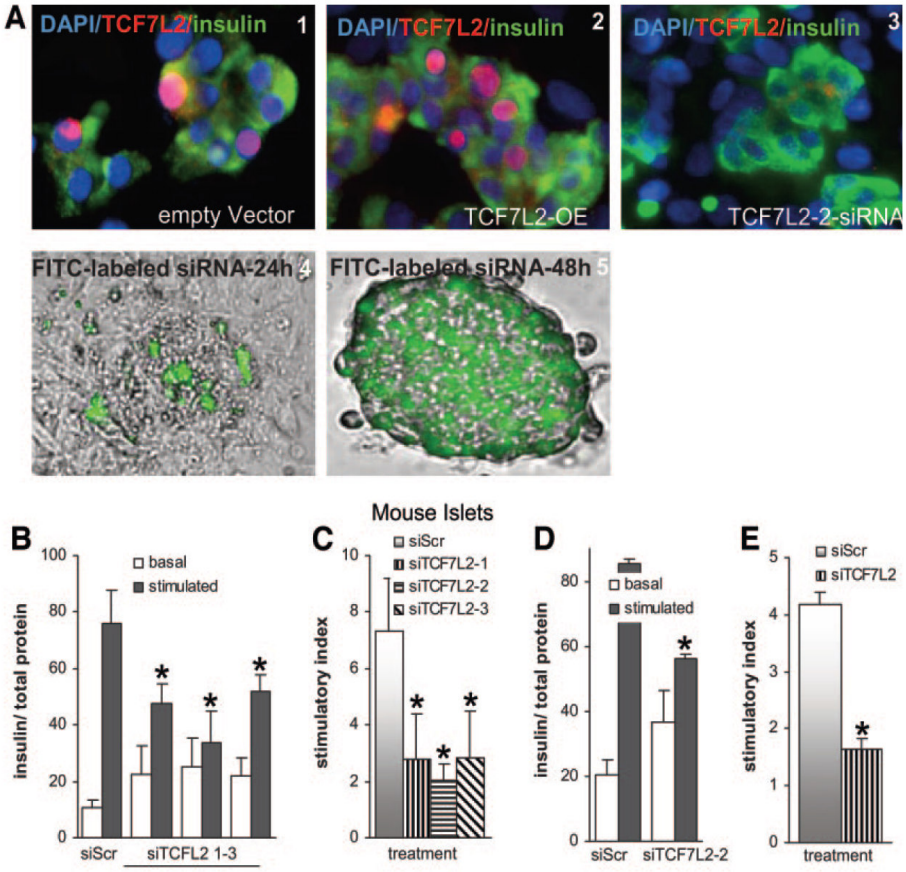

FIG. 1. TCF7L2 is expressed in the β-cell, and its depletion results in impaired β-cell function. *A*: Isolated human islets were cultured on extracellular matrix-coated dishes and transfected with an empty control plasmid (*1*), pCMV-TCF7L2 (*2*), or siRNA to TCF7L2 (*3*) for 4 days, fixed, and triple stained for TCF7L2 in red, DAPI in blue, and insulin in green. For evaluating transfection efficiency, islets were transfected with an FITC-labeled nontargeted siRNA and transfection was analyzed under the fluorescent microscope after 24 h (*4*) and 48 h (*5*). Isolated mouse (*B* and *C*) and human (*D–F*) pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to three different siRNA sequences to TCF7L2 (siTCF7L2-1, -2, and -3) or siScr for 4 days. *B* and *D*: Basal and stimulated insulin secretion indicate the amount secreted during 1-h incubations at 2.8 mmol/l (basal) and 16.7 mmol/l (stimulated) glucose after the 4-day culture period, normalized to whole islet protein content. *C* and *E*: Stimulatory index, which denotes the amount of stimulated divided by the amount of basal insulin secretion. *F*: Quantitative RT-PCR analysis of TCF7L2, insulin, and PDX-1 expression. The levels of mRNA expression were normalized against β-actin and tubulin with similar results and shown as change from control (siScr). Data represent results of two independent experiments from two different organ donors in quadruplicate (human) or from two independent experiments from mouse islets ($n = 8$). Results are means ± SE of siScr-treated controls at 5.5 mmol/l (human islets) or 11.1 mmol/l (mouse islets) glucose, *$P < 0.05$ to scrambled control. (Please see http://dx.doi.org/10.2337/db07-0847 for a high-quality digital representation of this figure.)

islets with siTCF7L2-2 only. Consistent with studies in mouse islets, GSIS was 1.5-fold decreased by siTCF7L2-2 in human islets ($P < 0.05$; Fig. 1*D*), and the stimulatory index was decreased 2.6-fold (Fig. 1*E*). These changes in insulin secretion were accompanied by a 54 and 69% decrease in human islet insulin and PDX-1 mRNA, respectively (Fig. 1*F*) and a 1.4-fold decrease in islet insulin content (17.6 pmol/islet in siScr-treated islets vs. 12.8 pmol/islet in siTCF7L2-treated islets, $P < 0.05$; data not shown).

We also performed islet perifusion studies in human isolated islets, which had been precultured for 4 days with siScr or siTCF7L2. Consistent with our results in the static incubation studies, depletion of TCF7L2 resulted in delayed and quantitatively diminished glucose-mediated insulin secretion ($P < 0.01$ at all time points; Fig. 2A). Furthermore, we examined GLP-1–induced stimulation of insulin secretion in control and siTCF7L2-treated islets (Fig. 2*A*). Depletion of TCF7L2 led to 5.4 ± 0.2-fold ($P < 0.001$) decreased ability of GLP-1 to stimulate GSIS in human islets. Finally, we examined β-cell maximal secretory capacity by exposing islets to perifusion medium containing 20 mmol/l KCl. Average KCl-induced insulin levels were 5.2-fold higher in the siScr-treated islets than in the siTCF7L2-treated islets ($P < 0.001$). However, the average stimulatory index from KCl >2.8 mmol/l glucose alone was similar in both treatment groups (2.2 in siScr and 2.3 in siTCF7L2, NS), suggestive of effective KCl stimulation in TCF7L2-depleted islets.

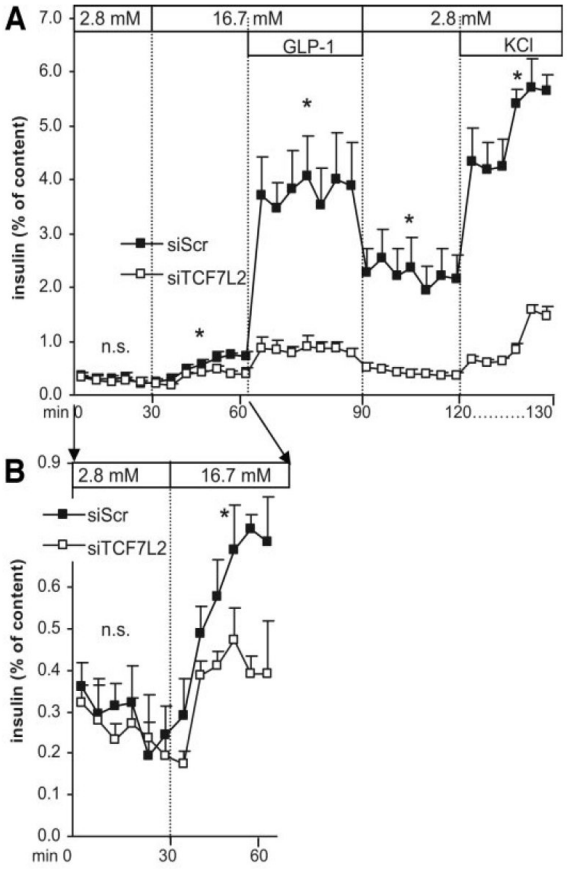

FIG. 2. Loss of TCF7L2 results in impaired glucose and GLP-1 stimulated insulin secretion. Human islets transfected with siScr or siTCF7L2-2 were placed in perifusion chambers in aliquots of 20 of equal size. The perifusate contained 2.8 mmol/l glucose for 1 h, 16.7 mmol/l glucose for 40 min, 16.7 mmol/l glucose and 100 nmol/l GLP-1 for 30 min, 2.8 mmol/l glucose for 30 min (the effluent was collected in 5-min intervals), and 20 mmol/l KCl for 10 min (the effluent was collected in 2-min intervals). Insulin secretion is expressed as percentage of content. One experiment was performed in triplicate from one organ donor. *A* and *B* show results from the same experiment with different *y*-axes scale to visualize changes in GSIS. *$P < 0.05$ to siScr control.

Depletion of TCF7L2 results in impaired β-cell turnover. Because β-cell apoptosis has been recognized as the underlying mechanism of β-cell destruction and consequent decrease in β-cell mass in type 2 diabetes (25), we tested whether depletion of TCF7L2 would also impair β-cell survival. Exposure of human islets to two different siRNA sequences to TCF7L2 resulted in a 2.2- and 2.5-fold decrease in β-cell proliferation, analyzed by double staining for Ki67 and insulin ($P < 0.001$; Fig. 3*A* and *C*) and 5.1- and 3.4-fold increase in β-cell apoptosis, analyzed by double staining for TUNEL and insulin ($P < 0.001$, Fig. 3*B* and *C*). Because the TUNEL assay also recognizes necrosis and DNA repair, we performed Western blot analysis for cleaved Caspase-3 and cleaved PARP, two of the most downstream products of apoptotic signaling. Together with a 2.4-fold downregulation in TCF7L2 protein expression, cleaved Caspase-3 was 5.2-fold and cleaved PARP was 2.1-fold higher expressed in human islets exposed to siTCF7L2 for 4 days (Fig. 3*D* and *E*). AKT is an important survival factor for β-cells. By Western blot analysis, we measured basal AKT phosphorylation in siScr- and siTCF7L2-treated islets. After a 4-day culture of human islets in 10% FCS and 5.5 mmol/l glucose, *p*-AKT was maintained but almost undetectable in the TCF7L2-depleted islets (Fig. 3*F*).

Overexpression of TCF7L2 protects from glucotoxicity and cytokine toxicity. Because the loss of islet TCF7L2 expression resulted in significant impairment of β-cell function and survival, we tested the hypothesis of whether environmental factors that contribute to β-cell failure in diabetes influence TCF7L2 expression levels and whether TCF7L2 overexpression can rescue from β-cell death.

We exposed isolated mouse and human pancreatic islets to increasing glucose concentrations (5.5, 11.1, and 33.3 mmol/l) and the mixture of cytokines (2 ng/ml IL-1β plus 1,000 units/ml IFN-γ) and transfected with either pCMV-TCF7L2 or an empty control plasmid under the same promoter. After the 4-day culture period, we performed GSIS (Fig. 4) and immunostaining for β-cell proliferation and apoptosis (Fig. 5). As we and others have reported before, increased glucose levels and exposure of islets to IL-1β plus IFN-γ impaired β-cell survival and function in mouse and human islets (26). Importantly, islets transfected with TCF7L2 were protected against the deleterious effects of glucose and of cytokines. The stimulatory index was 2.7-fold decreased by 33.3 mmol/l glucose and 7.3-fold by cytokines, compared with control incubations in mouse islets at 11.1 mmol/l alone ($P < 0.01$; Fig. 4*A* and *B*). TCF7L2 overexpression induced a 2.6-fold and 4-fold increase in stimulatory index at 33.3 mmol/l glucose and in islets treated with IL-1β plus IFN-γ, respectively, compared with control, protecting from the deleterious effects of both. Basal insulin secretion levels were increased by 33.3 mmol/l glucose and by cytokines in mouse islets (Fig. 4*A*), possibly as an indicator of the increased β-cell apoptosis. TCF7L2 overexpression reduced such an increase significantly ($P < 0.01$). Additionally, TCF7L2 increased stimulated insulin secretion in cytokine-treated islets, indicating its protective effect on β-cell insulin secretion and survival.

In human islets, similar protective effects were observed. Increased glucose levels (11.1 and 33.3 mmol/l) and the cytokine mixture IL-1β plus IFNγ reduced the islet stimulatory index 3.7-, 4.7-, and 3.2-fold, respectively ($P < 0.01$; Fig. 4*C* and *D*), compared with conditions at 5.5 mmol/l glucose. TCF7L2 overexpression improved β-cell GSIS significantly in all conditions. Interestingly, also at 5.5 mmol/l glucose, TCF7L2 improved stimulated insulin secretion (Fig. 4*C* and *D*). We observed similar protective results of TCF7L2 overexpression when we looked at β-cell apoptosis and proliferation. Elevated glucose concentration dose dependently reduced β-cell proliferation (2.5-, 5.2-, and 7.4-fold reduction by 11.1 and 33.3 mmol/l glucose and by IL-1β plus IFNγ, respectively; $P < 0.001$; Fig. 5*A* and *C*) and induced β-cell apoptosis (1.8-, 2.7-, and 1.6-fold induction by 11.1 and 33.3 mmol/l glucose and by IL-1β plus IFNγ, respectively; $P < 0.001$; Fig. 5*B* and *C*) in isolated human islets, compared with control incubations. This was reversed by TCF7L2 overexpression. Proliferation was 1.8-, 2.5-, and 3.1-fold induced (Fig. 5*A* and *C*) and apoptosis was 1.6-, 1.6-, and 1.5-fold reduced (Fig. 5*B* and *C*) by TCF7L2 at 11.1 and 33.3 mmol/l glucose and at IL-1β plus IFN-γ, respectively ($P < 0.01$). To investigate how chronically elevated glucose levels and cytokines, which

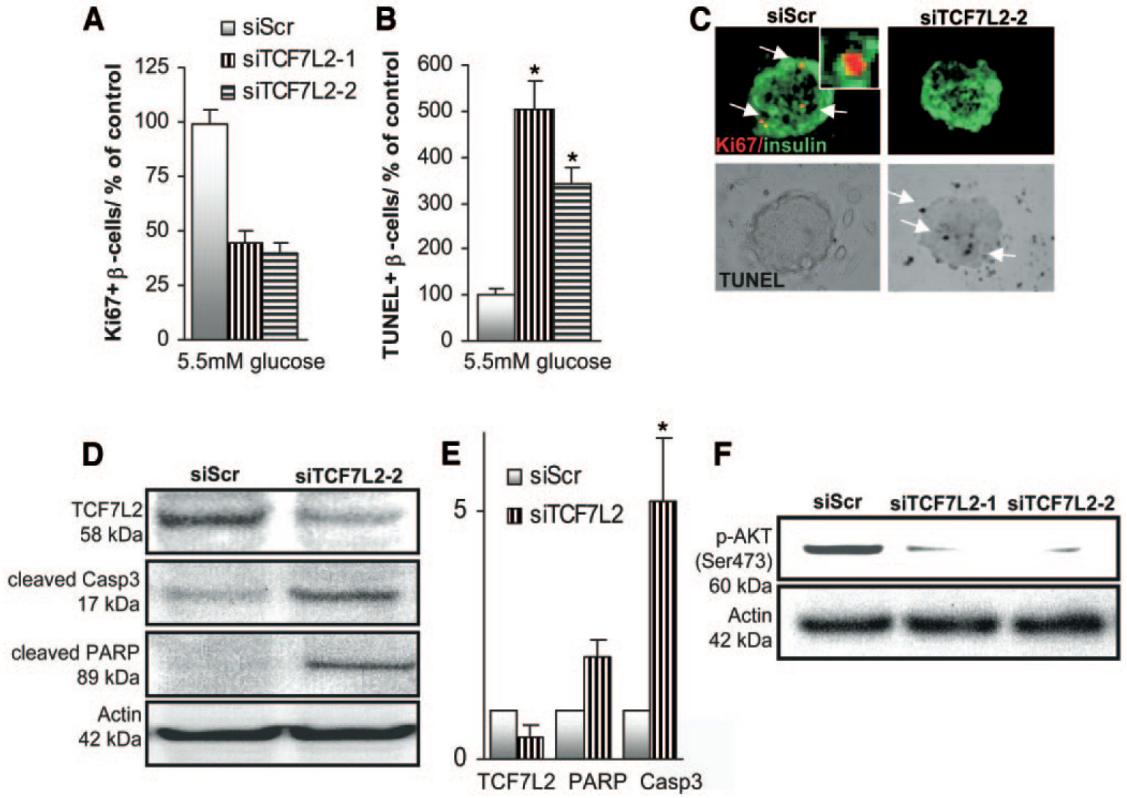

FIG. 3. Depletion in TCF7L2 results in impaired β-cell turnover. Isolated human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to two different siRNA sequences to TCF7L2 (siTCF7L2-1 and -2) or siScr for 4 days. Proliferation was measured by the Ki67 antibody (*A* and *C*, *top panel*) stained in red (cy3) and apoptosis by the TUNEL assay (*B* and *C*, *bottom panel*) and stained in black (alkaline phosphate). Islets were triple stained for insulin in green (*C*, *top panel*) and counterstained for DAPI in blue (not shown). White arrows point to proliferating and apoptotic β-cells. The insert of higher magnification shows a proliferating β-cell. Results are means ± SE of the percentage of Ki67-positive β-cells (*A*) or TUNEL-positive β-cells (*B*) normalized to control incubations (siScr) at 5.5 mmol/l glucose alone (100%; in absolute value, 0.29% TUNEL-positive and 1.78% Ki67-positive β-cells). The average number of β-cells counted was 26,130 for each treatment group in each of the two separate experiments from two different organ donors in quadruplicate ($n = 8$). *D* and *F*: Western blot analysis of TCF7L2, cleaved Caspase-3, cleaved PARP, Actin (*D*), and *p*-AKT and Actin (*F*) in human islets exposed for 4 days to siScr or siTCF7L2. Actin was used as a loading control on the same membrane after stripping. Blot is representative of three independent experiments from three different organ donors. *E*: The density of expression levels was quantified after scanning, normalized to actin levels, and expressed as change from siScr control, respectively. *$P < 0.05$ to untreated control. (Please see http://dx.doi.org/10.2337/db07-0847 for a high-quality digital representation of this figure.)

contribute to β-cell failure in diabetes, influence TCF7L2 expression levels and to correlate levels of TCF7L2 expression with apoptosis, we performed Western blot analysis from glucose/IL-1β plus IFN-γ–treated human islets transfected with pCMV-TCF7L2 or an empty control plasmid. Elevated glucose and IL-1β plus IFN-γ significantly downregulated TCF7L2 protein expression (Fig. 5*D*). In parallel to the 2.2- and 3.6-fold TCF7L2 downregulation by 11.1 mmol/l glucose or IL-1β plus IFN-γ, cleaved caspase-3 expression was 1.5-fold induced. In islets overexpressing TCF7L2, its protein expression levels were 1.5-, 2.4-, and 3.4-fold induced at 5.5 and 11.1 mmol/l glucose or 5.5 mmol/l glucose with IL-1β plus IFN-γ, compared with the empty vector–treated control, respectively (Fig. 5*D* and *E*; $P < 0.05$), and mRNA levels were 416-, 394-, and 705-fold increased, respectively ($P < 0.01$; data not shown). In contrast, caspase-3 was activated by glucose and IL-1β plus IFN-γ, and TCF7L2 reversed such upregulation. Even at control incubations, the signal for caspase-3, which is mostly seen in isolated human islets, was downregulated by TCF7L2 overexpression, indicating an overall protection from β-cell apoptosis by TCF7L2.

DISCUSSION

The identification of SNPs in the *TCF7L2* gene and its strong association with type 2 diabetes and with impaired insulin secretion (4–13) is an essential breakthrough in the field of genetics of type 2 diabetes.

Our study provides further evidence that TCF7L2 is an important regulator of β-cell function and survival. Chronic hyperglycemia and the cytokine mixture with IL-1β plus IFN-γ decreased TCF7L2 expression in the islets. In turn, TCF7L2 overexpression protected from the effects of chronically elevated glucose and cytokines on β-cell apoptosis and function. In vitro, TCF7L2 depletion in islets reduced proliferation, induced β-cell apoptosis, and decreased GSIS. During islet perifusion, insulin secretion in response to glucose was delayed and impaired in TCF7L2-depleted islets. Interestingly, GLP-1 increased

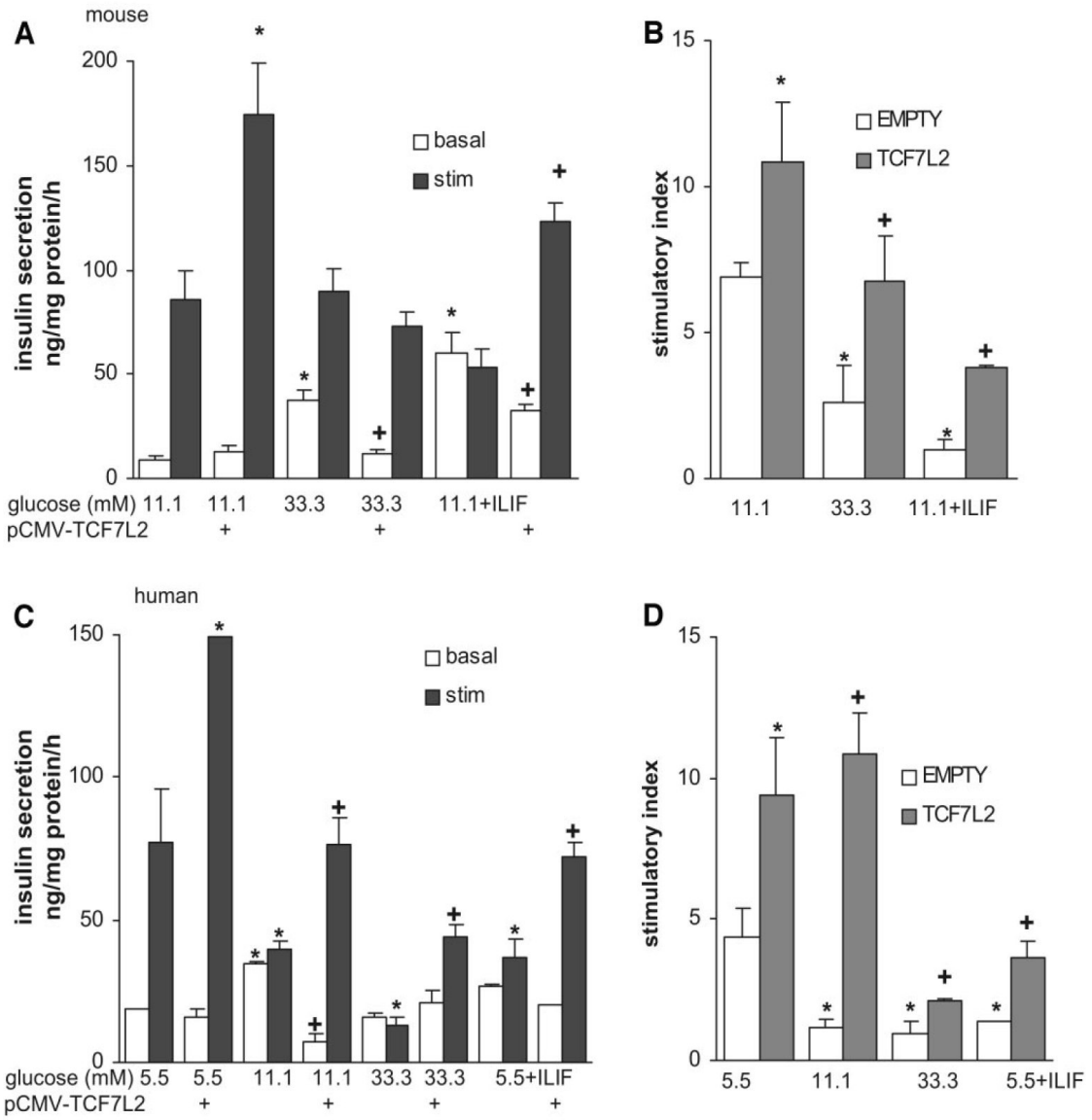

FIG. 4. Overexpression of TCF7L2 protects from glucotoxicity and cytokine toxicity. Isolated mouse (*A* and *B*) and human (*C* and *D*) pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to increasing glucose concentrations (5.5, 11.1, and 33.3 mmol/l) or to the mixture of IL-1β plus IFN-γ (ILIF) and transfected with either pCMV-TCF7L2 or an empty control plasmid under the same promoter. *A* and *C*: Basal and stimulated insulin secretion indicate the amount secreted during 1-h incubations at 2.8 mmol/l (basal) and 16.7 mmol/l (stimulated) glucose after the 4-day culture period, normalized to whole islet protein content. *B* and *D*: Stimulatory index, which denotes the amount of stimulated divided by the amount of basal insulin secretion. Data represent results of two different experiments from two different organ donors in quadruplicate (human) or of two independent experiments from mouse islets ($n = 8$). Results are means ± SE of empty vector–treated control islets at 5.5 mmol/l (human islets) or 11.1 mmol/l (mouse islets) glucose. *$P < 0.05$ to untreated control, +$P < 0.05$ to empty vector–transfected islets at same treatment.

GSIS in control islets to much greater extent than in TCF7L2-depleted islets. These data are in line with a recent study in human patients, which shows that carriers of the TCF7L2 risk allele showed a significant reduction in GLP-1–induced insulin secretion without defects in GLP-1 secretion during an oral glucose tolerance test (27). This supports the hypothesis that changes in TCF7L2 result in a functional defect of GLP-1 signaling in β-cells and may explain the impaired insulin secretion in carriers of the TCF7L2 risk alleles and the increased risk of type 2 diabetes. Cell membrane depolarization by addition of 20 mmol/l KCl raised insulin secretion in both control and TCF7L2-depleted islets. Although the absolute KCl-stimulated insulin secretion was much higher in control islets, the relative increase in insulin levels in response to KCl was comparable in both groups. Calculation of the average stimulatory index in islets in response to KCl showed no differences in control and TCF7L2-depleted islets. One

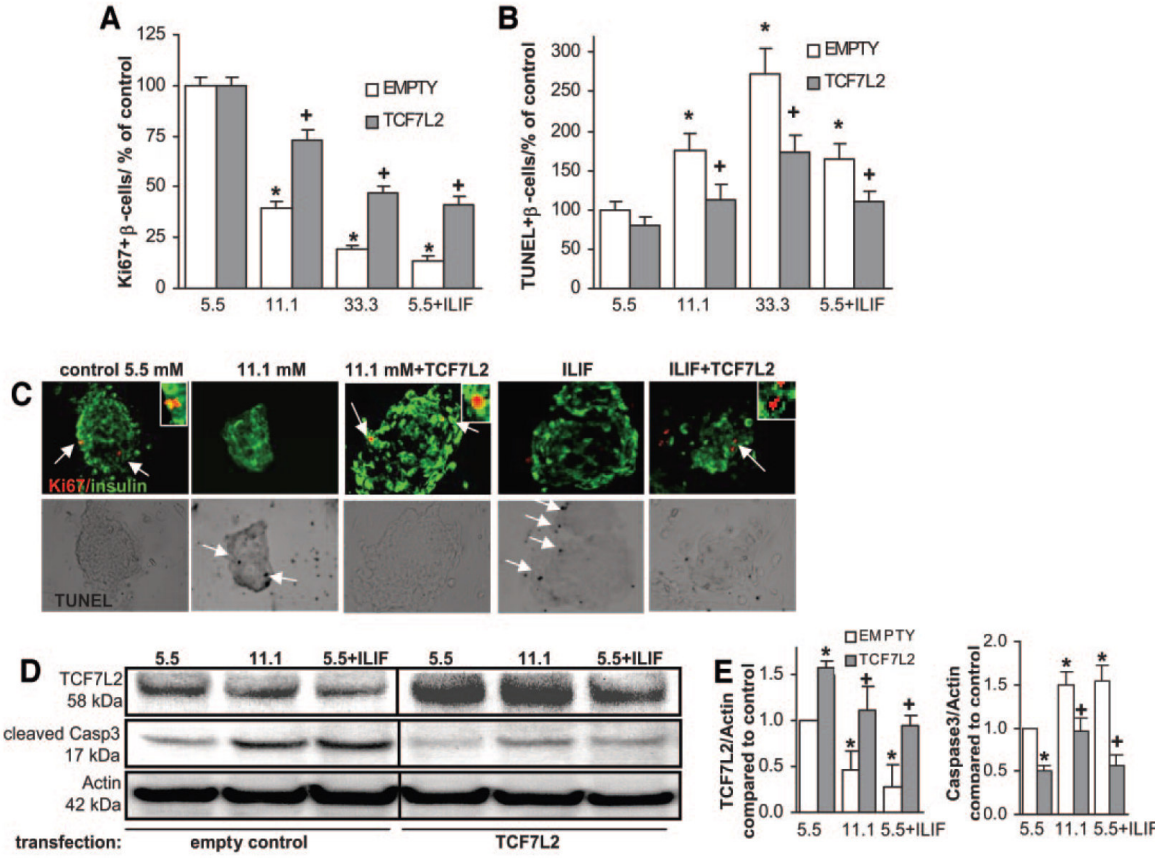

FIG. 5. Overexpression of TCF7L2 protects from glucose- and cytokine-induced β-cell apoptosis and decreased proliferation. Isolated human pancreatic islets were cultured on extracellular matrix-coated dishes and exposed to increasing glucose concentrations (5.5, 11.1, and 33.3 mmol/l) or the mixture of IL-1β plus IFN-γ (ILIF) and transfected with either pCMV-TCF7L2 or an empty control plasmid under the same promoter. Proliferation was measured by the Ki67 antibody (*A* and *C*, *top panel*) stained in red (cy3) and apoptosis by the TUNEL assay (*B* and *C*, *bottom panel*) and stained in black (alkaline phosphate). Islets were triple stained for insulin in green (*C*, *top panel*) and counterstained for DAPI in blue (not shown). White arrows point to proliferating and apoptotic β-cells. The insert of higher magnification shows a proliferating β-cell. Results are means ± SE of the percentage of Ki67-positive β-cells (*A*) or TUNEL-positive β-cells (*B*) normalized to control incubations (pCMV-empty) at 5.5 mmol/l glucose alone (100%; in absolute value, 0.33% TUNEL-positive and 2.09% Ki-67-positive β-cells). The average number of β-cells counted was 20,630 for each treatment group in each of the two separate experiments from two different organ donors in quadruplicate ($n = 8$). *D*: Western blot analysis of TCF7L2, cleaved Caspase-3, and actin in human islets exposed for 4 days to 5.5 or 11.1 mmol/l glucose or 5.5 mmol/l plus IL-1β plus IFN-γ. Islets were transfected with a CMV-TCF7L2 plasmid or an empty control plasmid using the same promoter (pCMV-empty). Actin was used as a loading control on the same membrane after stripping. Blot is representative of three independent experiments from three different organ donors. *E*: The density of expression levels were quantified after scanning, normalized to actin levels and expressed as change from control (5.5 mmol/l glucose). *$P < 0.05$ to untreated control, +$P < 0.05$ to empty vector–transfected islets at same treatment. (Please see http://dx.doi.org/10.2337/db07-0847 for a high-quality digital representation of this figure.)

important mechanism of the potentiation of GSIS by GLP-1 is the increase in the number of insulin secretory granules that dock to the β-cell membrane during stimulation (28). GLP-1 significantly increases the maximum insulin secretion induced by glucose and by KCl (29). Therefore we hypothesize that defective GLP-1 signaling observed in our study is responsible for the observed impaired absolute KCl-induced insulin response.

The protective effect of TCF7L2 overexpression on impaired β-cell function induced by glucotoxicity and cytokine toxicity was mainly a result of a decreased basal insulin secretion. Apoptotic cells have often increased basal insulin levels during the 1-h static incubation, as a result of dying cells, which release their insulin. Especially under conditions of chronic hyperglycemia, basal insulin release is increased (30). These data provide further evidence of the important role of TCF7L2 on β-cell survival.

It has been recognized in the past that isolated islets themselves are highly susceptible to β-cell apoptosis, whereas in vivo, only very few apoptotic cells can be found in pancreatic sections. For analysis of β-cell proliferation in this study, we used islets from young human islet donors (24 and 41 years) and therefore observed a relatively high number of proliferating cells when we plated the islets on extracellular matrix-coated dishes, with the majority being β-cells detected by double staining for Ki67 and insulin. This culture condition allowed us to study long-term islet survival and function, and we also detected some β-cell proliferation (21), whereas in vivo, β-cell proliferation in adult human islets is very limited. Using the same culture conditions, we have recently

shown that there is an age-dependent decline in β-cell proliferation (31), which was consistent with a study by Butler et al. (3) using pancreatic sections from autopsy.

One important regulator of β-cell survival is the protein kinase B/AKT pathway. Also the anti-apoptotic mechanism of GLP-1 is mediated through AKT (32). AKT activation has been reported to mediate survival of isolated human islets through insulin in an autocrine manner (33). Reduced AKT activity has also been associated with defective insulin secretion (34). Moreover, impaired AKT activation was associated with reduced GSK-3 inactivation (35), a gene associated with increased β-cell apoptosis (36) and compound in the WNT signaling pathway, which degrades β-catenin, and thus restricting nuclear activation of TCF7L2 i (37). Almost undetectable *p*-AKT in the TCF7L2-depleted islets therefore may explain the deleterious effects of loss of TCF7L2 on both β-cell survival and insulin secretion.

TCF7L2 is expressed in isolated human islets, shown by Western blot analysis, RT-PCR, and immunostaining. This is in line with recent results from human islets (10,38) and from Zucker diabetic fatty rat islets and their lean controls. In contrast, TCF7L2 was not detectable in mouse pancreatic sections and in the pancreatic InR1-G9 cell line (15).

In our study, we have observed that treatment of isolated islets with siRNA to TCF7L2 resulted in decreased TCF7L2 mRNA and protein expression and induced impaired β-cell survival and function. In contrast, TCF7L2 mRNA is increased in islets isolated from the Zucker diabetic fatty rat (38) and from patients with type 2 diabetes (12). Also, in individuals carrying an increased number of TCF7L2 risk T-alleles, TCF7L2 mRNA expression levels are increased (12). Neither study investigated TCF7L2 protein levels. Because we observed that increased TCF7L2 protein had protective effects on β-cell survival, we hypothesize that the posttranscriptional regulation of TCF7L2 rather than changes in mRNA levels may alter the β-cell.

TCF7L2 mRNA expression was also analyzed in muscle, fat, and lymphocytes in correlation with TCF7L2 variants. In transformed lymphocytes, TCF7L2 was differently regulated in controls and patients with type 2 diabetes; in control individuals, TCF7L2 mRNA expression decreased with the number of T-alleles, whereas in type 2 diabetes, it increased. Interestingly, no changes were observed in muscle or fat tissue (39), although TCF7L2 expression in adipose tissue is decreased in obese individuals with type 2 diabetes (10).

In this study, we report that elevated glucose levels and cytokine in cultured islets decreased TCF7L2 mRNA and protein levels. Therefore, further in vivo studies are needed using TCF7L2 variants in mice to confirm these results and translate them into the pathophysiology of type 2 diabetes.

Our data show that regulation of TCF7L2 plays an important role in the regulation of both β-cell survival and function and that targeting its expression could be a new strategy to maintain β-cell survival in diabetes.

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7.1.4 IP-10 is Produced by Human Pancreatic Islets in Type 2 Diabetes and Impairs β-Cell Function and Viability through TLR4 Signaling.

Schulthess FT, Sauter NS, Ribaux P, Haataja L, Strieter RM, Rizza RA, Oberholzer J, King CC, Maedler K.

Manuscript submitted

IP-10 is produced by human pancreatic islets in type 2 diabetes and impairs β-cell function and viability through TLR4 signaling

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Summary

In both, type 1 (T1DM) and type 2 diabetes (T2DM), β-cell destruction by apoptosis results in decreased β–cell mass and progression of the disease. We hypothesize that pro-inflammatory cytokines and chemokines contribute to the etiology. In the present study, we found that the Interferon-gamma-inducible Protein-10 (IP-10/CXCL10) plays a role in the progression of diabetes triggering impaired β-cell function and destruction.

Islets isolated from patients with T2DM secreted IP-10 and contained 33.5-fold more IP-10 mRNA than islets from control patients. Pancreatic sections from patients with T2DM and T1DM were found to express IP-10 in the β-cells.

Treatment of isolated human islets with recombinant human IP-10 induced β-cell proliferation and increased beta-cell apoptosis. Quantitatively, this resulted in a decrease in beta-cell viability. IP-10 induced changes in beta-cell turnover were accompanied by impaired glucose stimulated insulin secretion (GSIS) and decreased insulin mRNA. IP-10 induced rapid and sustained activation of Akt and JNK and downstream cleavage of p21-activated protein kinase 2 (PAK-2), switching Akt signals from proliferation to apoptosis. This was mediated through TLR4 signaling. Our data suggest that secretion of IP-10 by the β-cell in diabetes causes impaired β-cell function and survival.

Introduction

The ability of the β-cells to secrete adequate amounts of insulin to maintain normoglycemia depends on their function and mass. In both, type 1 and type 2 diabetes, the major mechanism leading to the decreased β-cell mass is increased β-cell apoptosis (1-3). In type 1 diabetes (T1DM), β-cell destruction occurs through immune mediated processes; mononuclear cell infiltration in the pancreatic islets and interaction between antigen presenting cells and T-cells leads to high local concentrations of inflammatory cytokines, chemokines, reactive oxygen species (ROS) and other inflammatory products (4; 5).

Type 2 diabetes (T2DM) occurs when β-cells fail to compensate adequately to increased insulin demand, most commonly as a consequence of obesity (6). The decline in β-cell function is accompanied by a deficit in β-cell mass. Several mechanisms have been postulated to contribute to increased β-cell apoptosis in T2DM. These include endoplasmic reticulum (ER) stress, hyperglycemia, hyperlipidemia, oxidative stress, islet amyloid polypeptide (IAPP) oligomers and activation of pro-inflammatory cytokines (7-10).

The role of inflammation in the pathogenesis of T2DM is supported by several recent prospective studies. Inflammatory markers are found in obesity, insulin resistance and

diabetes and link the pathology of the metabolic diseases (11). Low-grade inflammation and activation of the innate immune system can lead to β-cell failure in T2DM (12). Although the markers of immune activation are below the levels of acute inflammation, there are increases in acute phase proteins such as CRP and factors associated with endothelial activation including ICAM, VCAM and t-PA, cytokines (IL-1β, IL-6, TNFα) and chemokines, e.g. RANTES, MCP-1, IL-8 and IP-10 (13-15). Elevated cytokine levels lead to impaired β-cell survival and function, shown in numerous publications (16; 17).

Interferon-γ-inducible protein IP-10, also known as chemokine (C-X-C motif) ligand 10 (CXCL10), is a 10-kDa secreted protein produced in a variety of cells including endothelial cells, monocytes, fibroblasts and keratinocytes (18).

Increased levels of IP-10 have been detected in the serum of patients with T1DM (19; 20) and in patients with high risk for developing T1DM (19) as well as T2DM (13; 15). A recent report also found elevated IP-10 levels in serum of patients with T2DM (21). In animal models, β-cells secrete IP-10 during insulitis (22-25) and isolated human islets produce IP-10 in response to IFN-γ (25). Inhibition of IP-10 delayed immune mediated diabetes by suppression of insulitis and permitting β-cell proliferation (22). Furthermore, in mice deficient for the IP-10 receptor CXCR3, T-cell mediated diabetes is substantially delayed (24). Collectively these studies imply that IP-10 is important in mediation of β-cell apoptosis in T1DM and a potential candidate in T2DM. In order to address this possibility we first posed the question, is IP-10 expression increased in the islet in T1DM and T2DM? Having affirmed that postulate we investigated, if increased IP-10 expression in the islet in T2DM is secondary to most common metabolic milieu in T2DM, specifically increased glucose or fatty acid concentrations. Since the answer is negative, we further postulate that the expression of IP-10 may be a primary event in the evolution of β-cell dysfunction in T2DM. We therefore finally examined the effects of IP-10 on β-cell function and viability in human islets and the underlying mechanisms.

Materials and Methods

Animals. Ethical approval for mouse studies was granted by the UCLA Chancellor's Animal Research Committee in agreement to NIH animal care guidelines. C57BL/6J wild-type mice and TLR4 knockout mice (C57BL/10ScCr) were obtained from Jackson Laboratory (Bar Harbor, ME). Homozygous CXCR3 on a C57BL/6J background, produced by gene targeting as described previously (26), were kindly provided by Dr. Craig Gerard, Perlmutter Laboratory, Children's Hospital and Harvard Medical School, Boston, were. Animals were

housed at 22°C with a 12-h light-dark cycle (lights on at 07:00) and allowed free access to water and chow.

Islet isolation and culture. Human islets were isolated from pancreata of eleven healthy organ donors and three with T2DM at the University of Illinois at Chicago, the University Hospital of Zurich and the Diabetes Institute for Immunology and Transplantation, University of Minnesota as described previously (27). The islet purity was >95%, as judged by dithizone staining. When this degree of purity was not primarily achieved by routine isolation, islets were handpicked. To include only functional human islets for the study, islet quality was assessed by performing glucose stimulated insulin secretion and double staining of islets for apoptosis by the TUNEL assay and insulin after overnight incubation. Human islets from three isolations were purified into single β-cells and cultured as described (28). Mouse islets were isolated by bile duct perfusion and collagenase digestion as described (29). In all experiments, islets were pre-cultured for 24 hours after the isolation before the experiment. The islets were cultured on matrix-coated plates derived from bovine corneal endothelial cells (Novamed Ltd., Jerusalem, Israel), allowing the cells to attach to the dishes and spread (30) or in suspension culture dishes. Human islets were cultured in CMRL 1066 medium containing 5.5 mM glucose and mouse islets in RPMI 1640 medium containing 11.1 mM glucose, both supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS (Invitrogen Ltd., Carlsbad, CA), hereafter referred to as culture medium. Two days after plating, when most islets were attached and began to flatten, the medium was changed to culture medium containing 5.5 or 33.3 mM glucose with or without 0.1 ng/ml or 50 ng/ml recombinant human or mouse IP-10, 0.1 ng/ml or 50 ng/ml recombinant I-TAC, 50 ng/ml or 500 ng/ml Mig, 2 ng/ml recombinant human IL-1β, 1,000 U/ml recombinant IFN-γ, 100 U/ml TNF-α (all R&D Systems Inc., Minneapolis, MN), 100 nM Wortmannin, 50 μM LY294002 (Cell signaling, Beverly, MA), adenoviruses for dominant negative MyD88m (100 MOI, kindly provided by Professor Brian Foxwell, Imperial College, London, UK) or , 100 MOI of luciferase-control viruses (kindly provided by Dr Alan Karlson, Novo Nordisc), 5, 20 or 40 μM human islet amyloid polypeptide (h-IAPP, as described previously (31)), 100 ng/ml LPS or fatty acids (Sigma Chemical, St. Louis, MO; 0.5 mM palmitate or 0.5 mM palmitate plus 0.5 mM oleate). Fatty acids were dissolved at 10 mM in culture medium containing 11% fatty acid–free BSA (Sigma) under N_2-atmosphere, shaken overnight at 37°C, sonicated 15 minutes, and sterile-filtrated (stock solution) (32). For control experiments, BSA in the absence of fatty acids was prepared, as described above. Male Wistar rat islets were isolated and β-cells were sorted as described previously (33). β-cells were allowed to spread for 24h on 804G-extracellular matrix (34) and treated with or without 50 ng/ml of IP-10 for 24h.

CXCR3 neutralization. Isolated mouse islets were pre-cultured 1 hour prior to IP-10 treatment (0.1 ng/ml recombinant mouse IP-10) with an antagonistic goat anti-mouse CXCR3 antibody (10 μg/ml), which has been previously demonstrated a specific neutralizing capacity against mouse IP-10 (35). Control islets were treated with goat serum at the same concentration.

Neutralization of human IP-10. The recombinant hIP-10 neutralizing antibody (R&D Systems Inc., Minneapolis, MN) was used at 2 μg/ml. 50 ng/ml recombinant hIP-10 was incubated with the antibody for 30 minutes at room temperature. Following this pre-incubation period, the cytokine-antibody solution was added to isolated human islets and incubated for 4 days. Control islets were incubated with the antibody only.

Measurement of IP-10 release. IP-10 secretion into culture media of isolated islets from healthy and organ donors with T2DM was assessed with a Human Cytokine/Chemokine LINCO*plex* Kit (Linco Research, Inc., St. Charles, MO) according to the manufacturer's instructions.

Measurement of changes in intracellular Ca^{2+} concentration. INS-1 cells were seeded on Fluoro-dishes with Cover glass bottom (WPI, Sarasota, FL) and cultured overnight in RPMI 1640 medium containing 3 mM glucose without FCS. Cells were then loaded with 2.5 μM Fluo-3 AM by incubation for 30 min. in Krebs-Ringer bicarbonate buffer (KRB) buffer. After washing, intracellular Ca^{2+} was assayed after single stimulation with I-TAC or Mig. or 20 mM glucose (+control) in a perfusion chamber (37^0C, supplied with 95% air and 5% CO2) onto the stage of a laser confocal microscope (DMIRE2; Leica, Dearfield, IL). Fluo-3 was excited with 488 nm light supplied by an argon. The images with 630x magnification were acquired using a CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Volocity software (McBain Instruments, Chatsworth, CA).

β-cell apoptosis and replication. After washing with PBS, islets were fixed with 4% paraformaldehyde followed by permeabilisation with 0.5% Triton X-100. To quantify apoptosis, the free 3'-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, AP; Roche Diagnostics, Indianapolis, IN). For β-cell proliferation studies, a monoclonal antibody against the human Ki-67 antigen was used (Zymed, San Francisco, CA), followed by detection using the streptavidin-biotin-peroxidase complex. Subsequently, islets were incubated for 40 minutes at 37℃ with a guinea pig anti-insulin antibody (Dako, Carpinteria, CA), followed by detection using either the fluorescein-conjugated rabbit anti-guinea pig antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) or the streptavidin-biotin-peroxidase complex.

MTT assay. The MTT colometric assay (In Vitro Toxicology Assay Kit, Sigma, St Louis, MO) was performed to assess cell viability according to manufacturer's instructions. Rat insulinoma (RIN) cells, were seeded on a 96-well plate at 2.0 x 10^4 cells/well. The next day, the medium was replaced with fresh medium containing recombinant human IP-10 (0.1 and 50 ng/ml) or vehicle. Four days later, the cells were incubated for three hours in the presence of 1 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in RPMI culture media without phenol red. Mitochondrial dehyrogenase activity reduced the yellow MTT dye to a purple formazan, which was then solubilized with acidified isopropanol and absorbance was read at 570 nm with an ELISA plate reader. (Spectramax 250; Molecular Devices, Sunnyvale, CA). The background wavelength at 690 nm was substracted from the 570-nm measurement.

Immunohistochemistry. Islets were cultured in suspension and washed with PBS. Islets were fixed in Bouin's solution for 15 minutes and resuspended in 2% melted agarose in PBS, followed by short centrifugation and paraffin embedding. Human pancreatic tissue was obtained at autopsy from nondiabetic subjects (all with fasting plasma glucose <110 mg/dl, age 65±13, BMI 25±6), from patients with T2DM (poorly controlled type 2 diabetic patients, all with documented fasting plasma glucose >145 mg/dl, age 72±10, BMI 27±2). Autopsies were taken within 12 hours of death. Patients had no chronic glucocorticoid treatment, no signs of autolysis or pancreatitis were detected in the pancreas. A pancreas biopsy was obtained from an 89-year old newly T1DM diagnosed patient (with random blood glucose of 349 mg/dl, details were described recently (36)).

Islet and pancreatic sections were deparafinized in toluene, rehydrated in grades of alcohol, and washed in H_20. Slides were exposed to antigen-retrieval using antigen unmasking buffer according to the manufacturer's instructions (Vector Laboratories, Inc. Burlingame, CA). After antigen unmasking, the sections were cooled to room temperature, permabilized in 0.4% Triton X-100/TBS for 30 minutes, and blocked with 0.2% Tween 20/3% IgG-free BSA/2% Triton X-100/TBS. Primary antibodies were diluted in the blocking solution and the following dilutions: mouse anti-IP-10, 1:50; mouse anti-CXCR3, 1:100 (R&D Systems Inc.); guinea pig anti-insulin, 1:100 (Dako, Carpinteria, CA); rabbit anti-glucagon, 1:500 (Immunostar Inc., Hudson, WI); rabbit anti-somatostatin, 1:100; rabbit anti-CD3, 1:100 (Dako, Carpinteria, CA); rabbit anti-caspase-3, 1:50 (cleaved, Biocare medical, Concord, CA); rabbit anti-synaptophysin, 1:200; rabbit anti-chromogranin A, 1:250 (Chemicon, Temecula, CA). Donkey anti-mouse, anti-guinea pig or anti-rabbit derived secondary Antibodies conjugated to FITC or Cy3 were used at dilutions of 1:100 (Jackson ImmunoResearch Laboratories Inc.). Sections and cells were embedded in glycerol gelatin (Sigma) or Vectashield mounting media (Vector Laboratories, Inc. Burlingame, CA), which

visualized all cells by DAPI staining. Fluorescent slides were analyzed using a Leica DM600 microscope and images acquired using Openlab software.

Western blot analysis. Islets were maintained in culture in suspension in non-adherent plastic dishes in supplemented CMRL 1066 medium as described above. Two days after plating, medium was changed and groups of 150 islets were incubated in culture medium. At the end of the incubation, islets were washed in PBS and lysed for 40 minutes on ice in 40 µl lysis buffer containing 20 mM Tris acetate, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate and 10 mM β-glycerophosphate. Prior to use, the lysis buffer was supplemented with Protease - and Phosphatase – Inhibitors (Pierce, Rockford, IL). Equal amounts of protein of each treatment group were run on NuPAGE 4-12% Bis-Tris gels. Proteins were electrically transferred to PVDF filters and incubated with rabbit anti-phospho-Akt Ser473 (#9271), rabbit anti-Akt (#9272), rabbit anti-caspase-3 (#9961), rabbit anti-phospho-JNK Thr183/Tyr185, rabbit anti- JNK (#9252), rabbit anti-actin (#4967) (all from Cell signaling, Beverly, MA), rabbit anti-αPAK-2 (C-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by incubation with horseradish-peroxidase-linked anti-rabbit IgG peroxidase. The emitted light was captured on X-ray film after adding Immun-Star HRP Substrate (Bio-rad Laboratories, Inc.). As a positive control for cleaved PAK-2, we used Hamster Ovary Cells (CHO), cultured for 45 min at 44°C as described previously (37).

Protein Pull-down Assay. Dynabeads® M-270 Epoxy (Invitrogen) were coated with recombinant human IP-10 (R&D Systems Inc., Minneapolis, MN) for 20 hrs at 37°C with slow tilt rotation prior to incubation with the human islet cell lysates. After washing with PBS + 0.2% BSA, human islet cell lysates were added to the beads and incubated with tilting and rotation for 1 hour. Bound proteins were eluted in Laemmli buffer for SDS-PAGE. Whole cell lysates from cultured islets, eluted samples as well as follow-throughs were electrically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Inc.) and incubated with rabbit anti-TLR-4 (H-80, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CCR3 (H-52, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-CCR5 (eBioscience, San Diego, CA), rabbit anti-GPR1 (MBL, Woburn, MA) and biotinylated anti-IP-10 (R&D Systems Inc., Minneapolis, MN, to control for IP-10 binding to the beads) followed by incubation with horseradish-peroxidase-linked anti-rabbit or peroxidase-conjugated Streptavidin.

RNA extraction and quantitative reverse transcription-PCR (RT-PCR). Total RNA was isolated from the cultured islets as described previously (38). For quantitative analysis, we used the Light Cycler quantitative PCR system (Roche Diagnostics, Indianapolis, IN) with a commercial kit (Light Cycler-DNA Master SYBR Green I; Roche). Human primers used were

5’CTTCCAAGGATGGACCACAC3’ and 5’AGCAGGGTCAGAACATCCAC3’ (IP-10), 5’GCTTTGACCGCTACCTGAAC3’ and 5’ATGTGGGCATAGCAGTAGGC3’ (CXCR3), 5’CTGGGAGTTGTTCACTGGGT3’ and 5’GGATGGGGTTCAAGCAACTA3’ (GPR1), 5’TCCTTCTCTCTTCCTATCAATC3' and 5’GGCAATTTTCTGCATCTG3’ (CCR3), 5’TTCATTACACCTGCAGCTCTCATTT3’ and 5’TCACAGCCCTGTGCCTCTTCT3’ (CCR5), 5’AAGCCGAAAGGTGATTGTTG3’ and 5’CTGAGCAGGGTCTTCTCCAC3’ (TLR4), 5’CTACCTAGTGTGCGGGGAAC3’ and 5’GCTGGTAGAGGGAGCAGATG3’ (Insulin) and compared to the house keeping gene 5’AGAGTCGCGCTGTAAGAAGC3’ and 5’TGGTCTTGTCACTTGGCATC3’ (α-Tubulin) and 5’TCACCCACACTGTGCCCATCTACGA3’ and 5'CAGCGGAACCGCTCATTGCCA ATGG3’ (β-actin).

Small interfering RNA. A knock down of TLR4 protein levels was carried out using the small interfering RNA (siRNA) technique. SiRNA-Lipofectamine2000 complexes were prepared according to the manufacturer’s instructions (Liptofectamine2000; invitrogen) using 50 nM siRNA to TLR4 (RNAs of 25 nucleotides, designed to target human TLR4; Stealth Select^{TM} RNAi, invitrogen) and scramble siRNA (Ambion, Austin, TE). After 8 h overnight incubation, the transfection medium was aspirated and replaced by fresh culture medium with or without IP-10. To monitor transfection efficiency of siRNA into the islets, we transfected fluorescein labeled non-targeted siRNA (Cell signaling, Beverly, MA) and analyzed transfection under the fluorescent microscope during for 4 days of culture.

Glucose-stimulated insulin secretion (GSIS). For acute insulin release in response to glucose, islets were washed and preincubated (30 min) in KRB containing 2.8 mM glucose. The KRB was then replaced by KRB containing 2.8 mM glucose for 1 h (basal), followed by additional 1 h incubation in KRB containing 16.7 mM glucose. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content. Insulin was determined using a human insulin ELISA kit (Dako, Carpinteria, CA) or a mouse insulin ELISA kit (Alpco, Windham, NH).

Statistical analysis. Samples were evaluated in a randomized manner by a single investigator (F.T.S.) who was blinded to the treatment conditions. Data are presented as means +/- SE and were analyzed by paired, Student’s *t* test or by analysis of variance with a Bonferroni correction for multiple group comparisons.

Results

IP-10 is expressed and secreted in isolated pancreatic islets from organ donors with T2DM

Healthy human islets neither secreted nor expressed IP-10 (Fig. 1A, 1B panel 1). In contrast, human isolated islets from organ donors with T2DM secreted 65.62 ± 14.6 pg/ml/20 islets within 24 hours, which was accumulated after 96 hours (196 ± 58 pg/ml/20 islets; Fig. 1A, $p<0.001$ vs. control islets). As a positive control for IP-10 secretion we incubated healthy islets in the presence of the cytokine IFN-γ (1000 U/ml). These islets secreted 999.1 ± 127.2 pg/ml IP-10/20 islets into the culture medium during the 24-hour culture period.

The expression of IP-10 mRNA by T2DM islets was confirmed by quantitative RT-PCR demonstrating a 33.5-fold increase compared to healthy controls ($p<0.05$, Fig. 1C). We also investigated the islet cellular localization of IP-10 in pancreatic sections obtained at autopsy from eight poorly controlled type 2 diabetic patients, all with documented fasting plasma glucose >145 mg/dl as well as from one pancreas biopsy obtained from a patient newly diagnosed with T1DM. Double immunostaining for IP-10 in red (Fig.1B panel 1,3,5) and insulin in green (Fig.1B panel 2,4,6) revealed the presence of IP-10 localized in the β-cells in human pancreatic sections from patients with T2DM (Fig.1B panel 3,4) and T1DM (Fig.1B panel 5,6), but not in non-diabetic controls (Fig.1B panel 1,2). The duration of T2DM in the different patients ranged from 1-26 years (table 1), and we did not detect differences in IP-10 expression among them. IP-10 was absent in α and δ cells, as assessed by double immunostaining with anti-IP-10 and anti-glucagon or anti-somatostatin antibodies, respectively in controls, T1DM or T2DM (data not shown). IP-10 was also undetectable in exocrine tissue. Isolated human islets exposed to IFN-γ stained positive for IP-10 (data not shown).

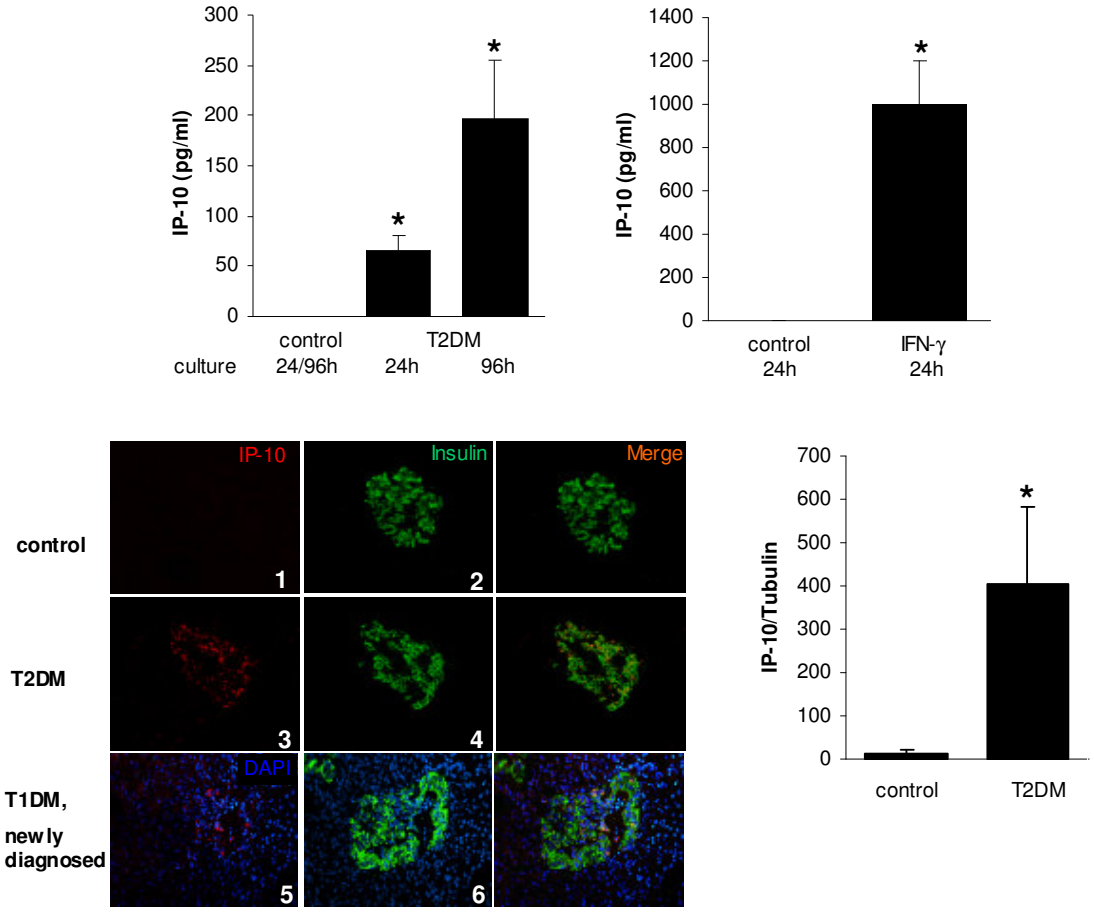

Figure 1-1. IP-10 is increased in islets from patients with T2DM.

(A) IP-10 secretion from isolated human pancreatic islets from five healthy control donors and three donors with T2DM cultured on extra- cellular matrix-coated dishes for 24 or 96 hours at 5.5 mM glucose (left) with or without addition of 1000 U/ml IFN-γ (right). **(B)** Double/ triple immunostaining for IP-10 in red (1,3,5) and insulin in green (2,4,6) and DAPI in blue in pancreatic tissue sections from a representative non-diabetic control (1,2) and from a patient with T2DM (3,4) or T1DM (5,6). (magnification x200). **(C)** RT-PCR quantification of IP-10 mRNA expression in human islets. The levels of IP-10 expression were normalized against tubulin. Results represent means ± SE from four non-diabetic controls and three organ donors with T2DM.

To investigate the processes that mediate IP-10 expression *in vitro*, human islets from non-diabetic organ donors were exposed for 48 hours to a diabetic milieu (0.5 mM palmitate, 0.5 mM palmitate plus 0.5 mM oleate, 2 ng/ml IL-1β 1,000 U/ml IFN-γ at 5.5 or 33.3 mM glucose (Fig. 1D left panel), the combination of the three cytokines 2 ng/ml IL-1β plus 1,000 U/ml IFN-γ plus 100 U/ml TNF-α (Fig. 1D middle panel) or for 24 hours to 20 or 40 µM h-IAPP or 100 U/ml TNF-α alone (Fig. 1D right panel). Western blot analysis revealed that IP-10 expression was independent of glucolipotoxicity or h-IAPP. IP-10 expression was induced by TNF-α and by IFN-γ (Fig.1D), confirming our RT-PCR and immunostaining results (not shown) and by the cytokine mixture IFN-γ plus IL-1β (Fig. 1D middle). Addition of TNF-α potentiated IP-10 expression induced by IFN-γ plus IL-1β. In contrast, elevated glucose levels did not further induce IFN-γ induced IP-10 expression (Fig. 1D lower panel).

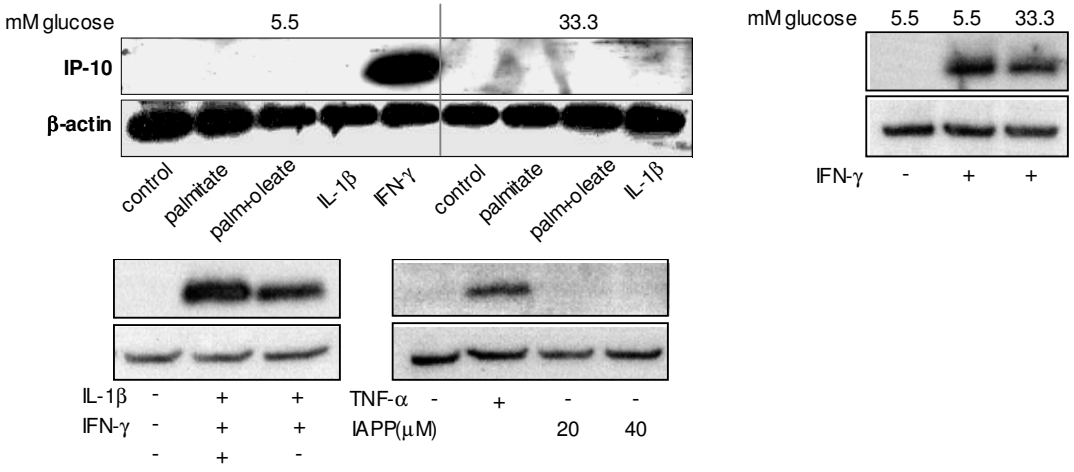

Figure 1-2. IP-10 is increased in islets from patients with T2DM.

(D) Human islets were cultured at 5.5 or 33.3 mM glucose for 48 hours with or without 0.5 mM palmitate, a mixture of 0.5 mM palmitate and 0.5 mM oleate, 1000 U/ml IFN-γ (left), with 2 ng/ml IL-1β plus 1000 U/ml IFN-γ alone or with 100 U/ml TNF-α (middle), with 1000 U/ml IFN-γ at 5.5 and 33.3 mM glucose (lower panel) or for 24 hours with 20 or 40 µM h-IAPP or TNF-α alone (right). Representative Western Blots out of six experiments from six healthy organ donors are shown. Actin was used as a loading control. The same membranes were incubated with IP-10 and actin antibodies after stripping. *$p < 0.05$ vs. healthy untreated control islets.

IP-10 induces β-cell death and proliferation and impaired β-cell function

To investigate the consequence of IP-10 expression in human type 2 diabetic islets, we added exogenous human recombinant IP-10 to cultured islets from healthy organ donors. We used 0.1 ng/ml IP-10, a concentration we measured from islets from donors with T2DM, as well as 50 ng/ml, the ED_{50} for the ability of IP-10 to chemo-attract T-lymphocytes cultured in the presence of IL-2 (39). Both 0.1 and 50 ng/ml IP-10 increased β-cell apoptosis by 1.6 ± 0.3-fold (n.s.) and 2.0 ± 0.4-fold ($p<0.01$) after 1 day (not shown) and 2.8 ± 0.5-fold and 2.9 ± 0.4-fold ($p<0.01$), respectively after 4 days of culture compared to untreated control islets at 5.5 mM glucose (Fig.2A,C). After 1 day of culture, IP-10 showed tendency to induced β-cell proliferation (n.s., data not shown) which was significant after 4 day-treatment (1.5-fold with 0.1 ng/ml and 2.7-fold with 50 ng/ml, $p<0.05$) compared to controls (Fig. 2B,D).

To verify a specific IP-10 mediated effect, human recombinant IP-10 was pre-incubated for 30 min with a neutralizing IP-10 antibody and then exposed to the islets. Treatment with anti-IP-10 Ab significantly inhibited IP-10 induced β-cell apoptosis after 4 days (1.5 fold, $p<0.05$) compared to IP-10 treated cells alone (Fig. 2E).

To confirm that IP-10`s actions were mediated by apoptosis and to investigate a matrix independent effect, we quantified cleavage of caspase-3. Human islets were cultured in suspension with or without IP-10. After the 48-hour treatment period, islets were Bouin's fixed, paraffin embedded and islet sections were double stained for cleaved caspase-3 and insulin. IP-10 induced a 2.6- ($p<0.05$) and 3-fold ($p<0.001$) increase in caspase-3 positive β-cells at 0.1 and 50 ng/ml IP-10 respectively (Fig. 2F). In order to quantify β-cell number after IP-10 exposure, we measured β-cell viability in the β-cell line RIN5m and detected an 18% and 25% decrease in viability by 0.1 and 50 ng/ml IP-10, respectively ($p<0.01$, Fig.2G).

IP-10-induced a 1.9-fold decrease in GSIS after long-term culture with IP-10, compared to control ($p<0.05$, Fig. 2H,I). To determine the acute effect of IP-10 on GSIS we cultured the islets in the presence of IP-10 at low and high glucose for 1 hour. At low glucose IP-10 had no significant effect, however at high glucose IP-10 decreased GSIS 1.8-fold compared to control ($p<0.05$), suggesting that even in short term IP-10 inhibits β-cell function (Fig.2J). Furthermore, insulin mRNA was 2.1 and 2.3–fold decreased after 1 day of culture by 0.1 and 50 ng/ml IP-10, respectively ($p<0.05$, Fig.2K).

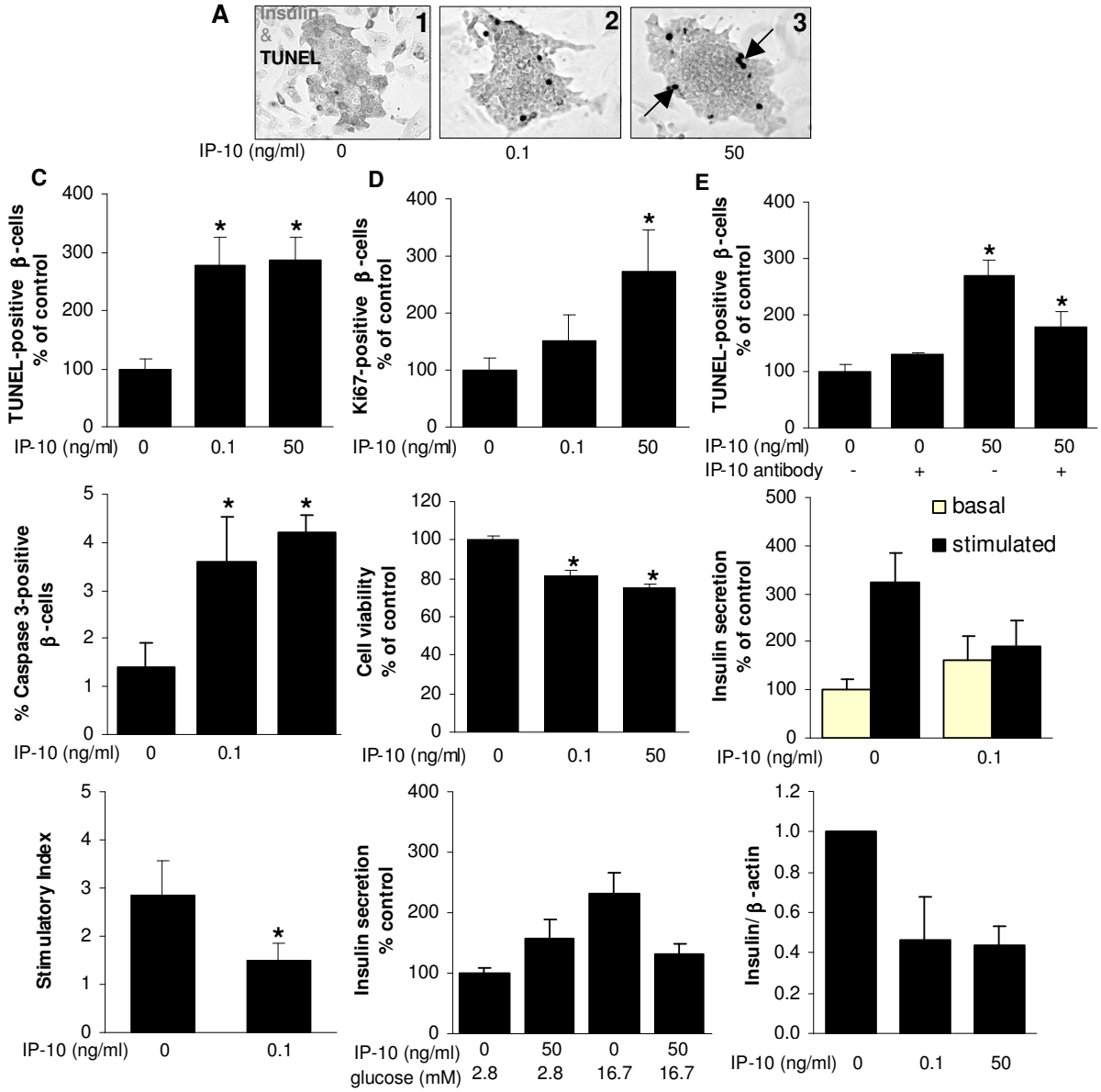

Figure 2. IP-10 induces β-cell death and impaired β-cell function

Human islets were cultured on ECM dishes for 4 days in 5.5 mM glucose alone or with increasing IP-10. Apoptosis was measured by the TUNEL assay (**A,C,E**) and stained in black (alkaline phosphate**, A1-3**) and proliferation by the Ki-67 antibody (**D**). Islets were double-stained for insulin in red (here grey, A1-3). Black arrows point doublets of apoptotic β-cells indicative of post-mitotic apoptosis **(A3)**. Results are means ± SE of the percentage of TUNEL-positive **(C,E)** or Ki-67 positive **(D)** β-cells normalized to control incubations at 5.5 mM glucose alone (100%; in absolute value: 0.46% TUNEL-positive and 0.33% Ki-67-positive β-cells) from four different experiments in triplicate from four different organ donors. (E) Human recombinant IP-10 was pre-incubated for 30 min with an antagonistic IP-10 antibody and then incubated with the islets. **(F)** Caspase-3 activation was analyzed in Bouin's-fixed paraffin embedded sections from human islets cultured in suspension for 48 hours with or without IP-10. Results are means ± SE of the percentage of cleaved caspase-3-positive cells. Each experiment was repeated three times from three organ donors. *$p < 0.05$ to untreated control. **(G)** MTT assay was performed on RIN cells exposed for 4 days to IP-10. Each bar represents the mean of three separate experiments ± SE. **(H-J)** GSIS from islets exposed to IP-10. **(G)** Basal and stimulated insulin secretion indicate the amount secreted during 1-hour incubations at 2.8 (basal) and 16.7 mM (stimulated) glucose following the 6-day culture period, normalized to insulin content and expressed as percent change from basal secretion. **(I)** Stimulatory index, which denotes the amount of stimulated divided by the amount of basal insulin secretion. **(J)** Acute basal and stimulated insulin secretion with or without IP-10 at low and high glucose for 1 hour, normalized to insulin content and expressed as percent change from basal secretion. **(K)** Quantitative RT-PCR analysis of insulin expression. The level of insulin mRNA expression was normalized against β-actin. Results were expressed as mRNA levels relative to control incubations. Data represent results of three different experiments from three different organ donors. Results are means ± SE of untreated controls at 5.5 mM glucose, *$p<0.05$ to untreated control.

We then examined the effects of two other ligands of CXCR3; IFN-γ inducible chemokines I-TAC (CXCL11) and Mig (CXCL9) on β-cell turnover in human isolated islets. In contrast to IP-10, neither I-TAC nor Mig at comparable concentrations based on their activity affected β-cell apoptosis and β-cell proliferation (Fig.3A) after 4 days of culture. To investigate a functional response induced by I-TAC and Mig, the mobilization of intracellular calcium was assessed by Fluo-3 in INS-1 cells after addition of the two chemokines. Both I-TAC and Mig induced the mobilization of $[Ca^{2+}]i$ in the cells, which confirms their activity (Fig.3B). As positive control for $[Ca^{2+}]i$ influx in the β-cells we used 20 mM glucose, which also resulted in Fluo-3 uptake.

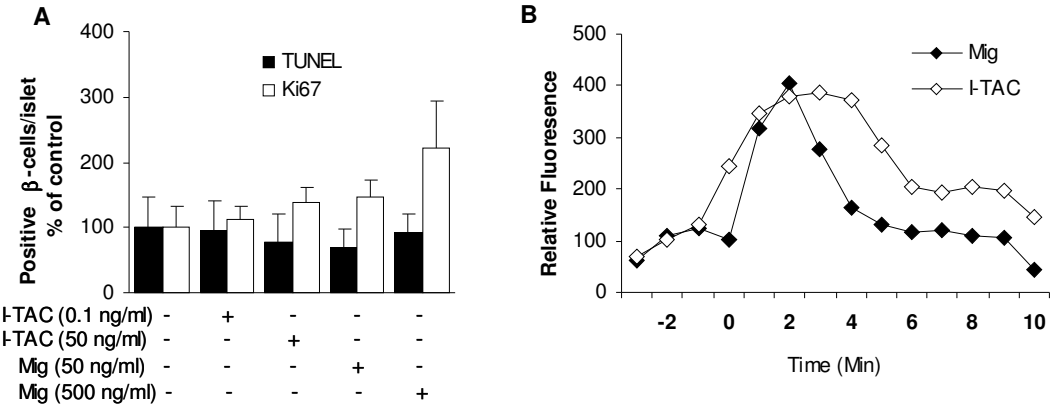

Figure 3. Distinct effects of other CXCR3 ligands

(A) Relative number of TUNEL-positive and Ki-67-positive □-cells after 4-days of culture in control conditions (5.5 mM glucose) with or without I-TAC (0.1-50 ng/ml) and Mig (50-500 ng/ml). Data are expressed as % of control conditions (100%; in absolute value: 0.10% TUNEL-positive or 0.16% Ki-67 positive □-cells) from three different experiments in triplicates from three different organ donors. (B) Fluo-3-uptake in the β-cell line INS-1 before and during 10 min of addition of Mig (full diamonds) or I-TAC (open diamonds), analyzed by on-line confocal microscopy (Leica). Data show relative fluorescence from one representative analysis from three independent experiments.

IP-10 mediated PAK-2 cleavage switches Akt signaling into apoptosis

Next we examined the molecular mechanism of the deleterious effects of IP-10 on cultured human islets. Since exposure to IP-10 led to both increased apoptosis and β-cell proliferation, we hypothesized, that IP-10 acts to drive β-cells into cell cycle, but these cells are then driven to apoptosis. By western blot analysis, we detected increased Akt phosphorylation within 30 minutes of culture with IP-10, which was sustained through the entire 24-hour culture period (Fig. 4A,B). The PI3K inhibitor Wortmannin (100 nM) blocked this Akt phosphorylation in the presence of IP-10, confirming PI3K signaling was involved (Fig.4A). Furthermore, PI3K inhibition fully blocked IP-10 induced β-cell death (Fig. 4C). Here, we show for the first time, that sustained Akt activation can trigger apoptosis in the β-cell. One of the direct downstream effectors of Akt is the p21-activated kinase 2 (PAK-2). Akt-mediated phosphorylation of PAK-2 mediates cell survival, however if PAK-2 is proteolytically cleaved, apoptosis is induced (40).

We found full length PAK-2 constitutively expressed in human islets and rat β-cells. However, upon IP-10 induced caspase-3 cleavage (Fig.2E for human islets & 4E for rat β-cells), we detected increased levels of the cleaved C-terminal fragment of PAK-2 (PAK-2p34) in human islets and in purified rat β-cells (Fig. 4D, E). Heat-shocked Hamster Ovary Cells (CHO), were used as positive control for PAK-2 cleavage (37). Besides caspase-3 and PAK-2 activation, IP-10 also triggered JNK activation, a pathway, which is activated by inflammatory cytokines and free fatty acids in the β-cell leading to β-cell apoptosis (41). After 5 minutes of exposure, IP-10 induced JNK activation that still persisted after 1 day of culture (Fig. 4F).

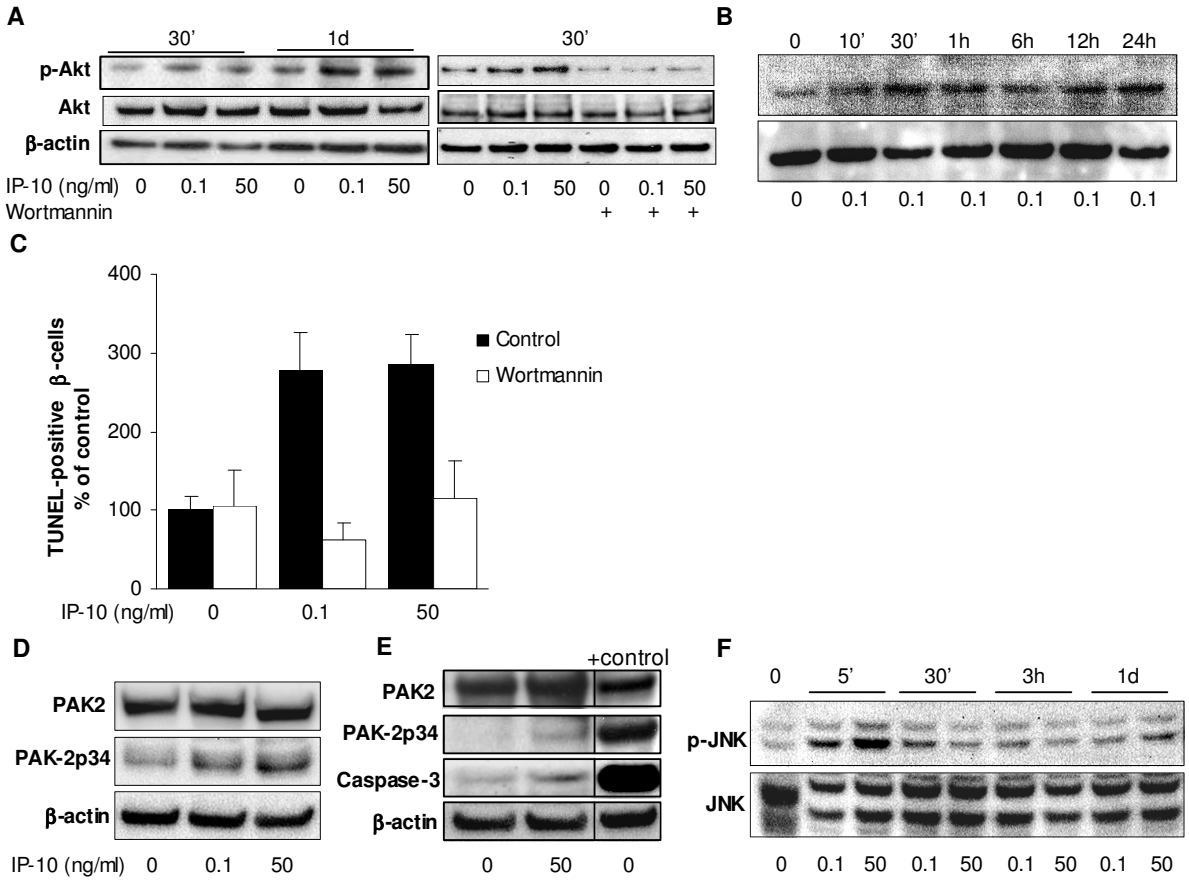

Figure 4. Activation of the PI3K/Akt pathway by IP-10 subsequently leads to PAK-2 cleavage and apoptosis

(A) Western blot analysis of p-Akt in human islets exposed for 30 min or 1 day to IP-10 without Wortmannin (100 nM) and **(B)** shows a time course experiment from 0 to 24 h after IP-10 treatment. Akt and Actin were used as loading controls. This Western blot is representative of four experiments from four different organ donors. **(C)** Human islets were cultured for 4 days in 5.5 mM glucose alone (control) or in the presence of IP-10 with or without addition of 100 nM Wortmannin. Results are mean ± SE of the percentage of TUNEL- positive β-cells normalized to control. *p< 0.05 relative to islets cultured at 5.5 mM glucose alone, $^{+}$p< 0.05 relative to islets in the absence of Wortmannin at the same IP-10 concentration. **(D)** Western blot analysis of full length PAK-2, cleaved PAK-2p34 and actin from human isolated and **(E)** of full length PAK-2, cleaved PAK-2p34 and activated caspase-3 from purified rat □-cells. Human islets and purified rat β-cells were cultured for 1 day in with or without IP-10. Heat shock (Hs) treated CHO cells were used as a positive control for cleaved PAK-2p34. Hs treatment was performed at 44°C for 45 min, followed by a 2h incubation at 37°C. Antibodies were incubated with the same membrane after stripping. **(F)** Human islets were stimulated with IP-10 for 5 min. to 24 hours. Total JNK was used as a loading control. One representative Western blot is shown out of four experiments from four different organ donors **(D,F)** or three different β-cell isolations **(E)**.

The effect of IP-10 on β-cells is independent of the CXCR3 receptor

IP-10 is the ligand for the receptor CXCR3. To further study the mechanisms of IP-10 induced β-cell destruction, we investigated the role of its receptor CXCR3. We exposed islets isolated from CXCR3 knockout mice to IP-10. Similar to our observations in human islets, IP-10 induced β-cell apoptosis in islets from wildtype C57Bl/6J mice (Fig. 5E). Furthermore, we did not detect alterations of IP-10 induced increase in β-cell death (Fig. 5A,B) and insulin secretion (Fig. 5C,D) in islets isolated from CXCR3 knockout mice (0.1 and 50 ng/ ml IP-10 increased β-cell apoptosis 2.7-fold and 2.0-fold, respectively ($p<0.05$) and stimulatory index was 3-fold decreased by 0.1 ng/ml IP-10 compared to control ($p<0.05$). Furthermore, we pre-cultured isolated pancreatic islets from C57Bl/6J mice with goat anti-mouse CXCR3, an antagonistic antibody to CXCR3, which has been shown previously to block IP-10 mediated effects (42). After 1 hour pre-culture, IP-10 was added to the islets for the 3-day culture period. IP-10 increased apoptosis 2.4-fold without and 2.6-fold with CXCR3 neutralization, compared to serum treated control (Fig. 5E, $p<0.05$). In parallel, proliferation was 1.7-fold increased without and 1.6-fold increased with CXCR3 neutralization, compared to serum treated control (Fig. 5F, $p<0.05$). There were no differences between the treatment groups with or without the neutralizing antibody.

Since IP-10 exhibits similar effects in the presence and absence of CXCR3, we assume that its effect is CXCR3-independent in pancreatic β-cells.

Subsequently, we analyzed CXCR3 gene expression in human islets. CXCR3 mRNA was detected in islets as well as in purified β-cells (Fig.5G). Sequence analysis of the PCR products confirmed CXCR3 expression according to published sequences. By fluorescent immunocytochemistry we found CXCR3 expression in human islets (1.08 ± 0.16 positive cells/islet). We observed CXCR3 positive β-cells (Fig.5H panel 1,2), but most CXCR3 positive cells were negative for insulin. No CXCR3 staining was observed in α and δ cells, as assessed by double immunostaining with anti-glucagon or anti-somatostatin antibodies, respectively (Fig.5H panels 3-6). Synaptophysin and chromogranin A (alternative markers for endocrine cells) only partially colocalized with CXCR3 (data not shown). Since CXCR3 is expressed in activated T-cells, we performed triple staining for insulin (Fig.5H panel 7), CXCR3 (Fig.5H panel 8) and a T-cell marker CD3 (Fig.5H panel 9) and found CXCR3 positive T-cells in human islets. However, we detected large variation in the number of these CXCR3 positive β- and T-cells in the six different human islet isolations studied. The expression levels of CXCR3 in the islets was similar in untreated and cells treated with IP-10 or IL-1β and IFN-γ (data not shown).

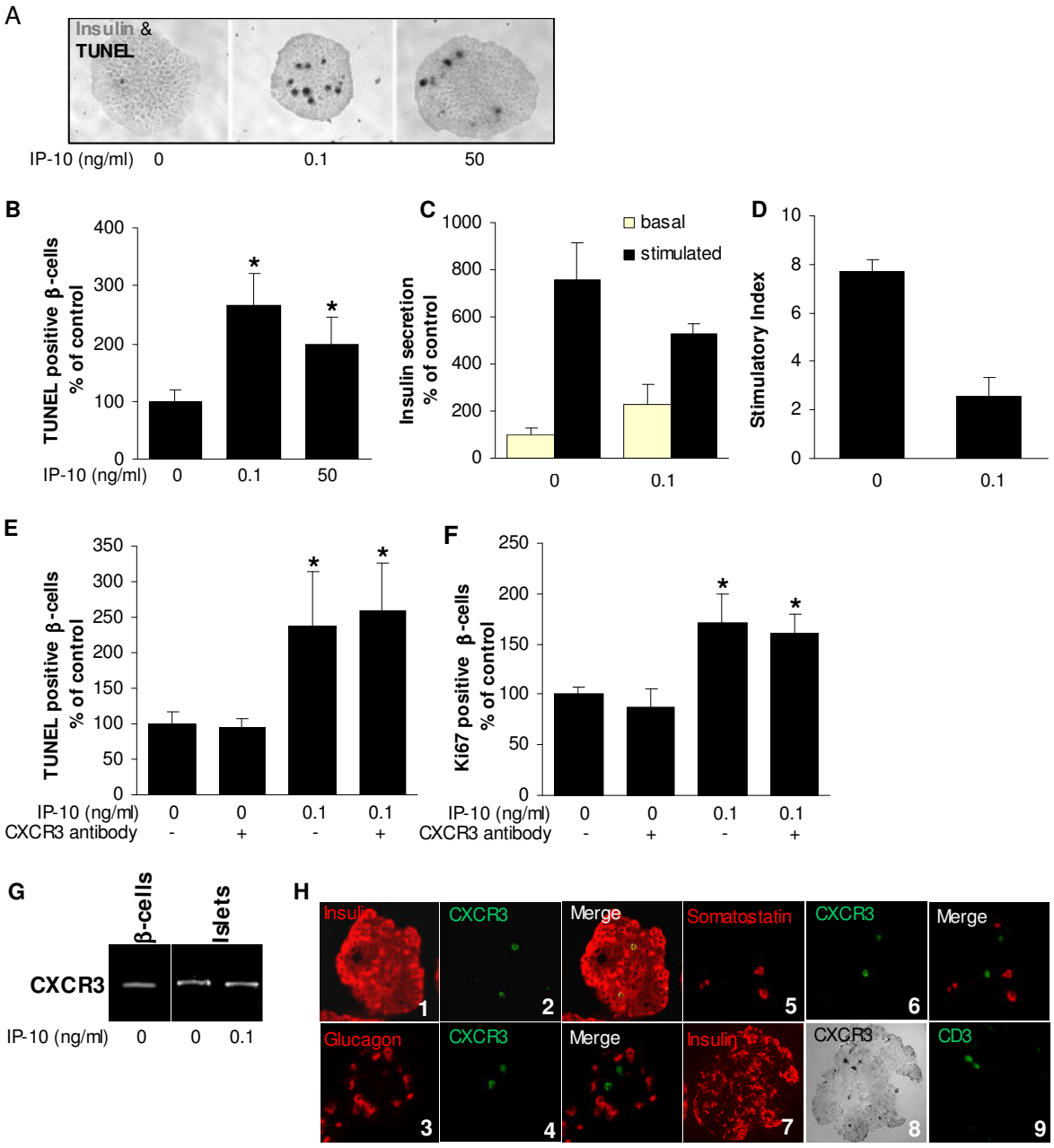

Figure 5. The effects of IP-10 are CXCR3 independent

(A-D) Analysis of β-cell apoptosis and function in CXCR3$^{-/-}$ mice. Mouse islets were cultured on extracellular matrix-coated dishes for 4 days in 5.5 mM glucose alone or in the presence of IP-10. **(A,B)** Islets were stained for TUNEL (black) and insulin (grey). Results are means ± SE of the percentage of TUNEL-positive β-cells versus control incubations at 5.5 mM glucose alone (100%; in absolute value: 1.12% TUNEL-positive β-cells). **(C)** Basal and stimulated insulin secretion during 1-hr incubation at 2.8 (basal) and 16.7 mM (stimulated) glucose following a 4-day culture period with or without IP-10, normalized to insulin content and expressed as percent change from basal secretion. **(D)** Stimulatory index. **(E,F)** Isolated islets from C57BL/6J mice were pre-cultured for 1 hr with goat serum (control) or with goat anti-mouse CXCR3 neutralizing antibody prior to addition of 0.1 ng/ml recombinant mouse IP-10. Results are means ± SE of the percentage of TUNEL-positive **(E)** and Ki-67-positive **(F)** β-cells versus control at 5.5 mM glucose. Results are means ± SE of two independent experiments in quadruplicates from six mice, respectively. *$p < 0.05$ to untreated control islets. **(G)** RT-PCR for CXCR3 was performed from purified human β-cells and human islets cultured for 3 days in 5.5 mM glucose alone or with IP-10. One of two experiments from two organ donors is shown. **(H)** Immunohistochemical analysis for CXCR3 on sections from human islets. Double/triple immunostaining for CXCR3 (green, 2,4,6, black, 8) and insulin (red, 1,7), glucagon (red, 3), somatostatin (red, 5) and CD3 (green, 9). Magnification x200.

IP-10 binds to TLR4 in pancreatic β-cells and mediates β-cell apoptosis

In the search for the specific receptor for the IP-10 mediated effects in β-cells, we analyzed gene expression of a number of receptors in islets by PCR. Initially, we looked for GPR1 (which is an orphan receptor, and has sequence motifs shared by chemokine receptors (43)), CCR3 (CXCR3 ligands were found to bind to CCR3 (44; 45), but exhibiting antagonistic effects), CCR5 which is highly up-regulated by TNF-α and IFN-γ, together with up-regulation of IP-10 (46), and TLR4, because upon its activation, IP-10 production is induced. Untreated human islets expressed all of the four receptors (Fig. 6A). To identify the binding capacity of IP-10 to these receptors beads were coated with IP-10 prior to incubation with human islet lysates. While CCR5 and GPR1 failed to bind IP-10 and CCR3 had a limited capacity to bind IP-10, we identified TLR4 as the receptor for IP-10 (Fig.6B). We next examined the ability of IP-10 to regulate TLR4 receptor expression. 0.1 ng/ml IP-10 caused a 2-fold induction of TLR4 mRNA (Fig.6C). Since TLR4 signaling results in up-regulation and secretion of IP-10 (47-49), we examined IP-10 production downstream of IP-10 signaling and measured a 4.5-fold and 33.3-fold induction of IP-10 mRNA in human islets after treatment with 0.1 and 50 ng/ml IP-10, respectively (Fig.6D). To investigate whether IP-10 signaling is TLR4 dependent, we tested the effect of IP-10 on islets isolated from TLR4-KO (8–12 weeks old) and measured β-cell apoptosis. We used lipopolysaccharide (LPS), the major ligand for TLR4 (50) as positive control to induce TLR4 dependent apoptosis. LPS induces β-cell damage in vitro (51) and in vivo (52). In our study, LPS induced a 2.9-fold induction of β-cell apoptosis, which was prevented in isolated islets from TLR4-KO mice (data not shown). IP-10 treatment induced a 2.2-fold induction of β-cell apoptosis in the wildtype islets, but not in the TLR4-KO islets (Fig.6E). To further confirm the involvement of TLR4 in mediating the effects of IP-10, we exposed human islets to a TLR4 specific siRNA (siTLR4) or scrambled control siRNA (siScr). We achieved an average down-regulation of 48% TLR4 protein expression by the siRNA, shown by Western blo analysis (Fig.6G). After a 4-day culture period with 0.1 ng/ml IP-10, we analyzed β-cell apoptosis. IP-10 treatment induced a 4-fold induction of β-cell apoptosis in human islets pre-treated with scramble control siRNA (siSCR). Pre-treatment with siTLR4 induced a 1.7-fold reduction of β-cell apoptosis ($p<0.05$, Fig.6F). We next investigated if IP-10 mediated TLR4 signaling is linked to the activation of the Akt/PAK-2/JNK pathway by Western blot analysis. Addition of a neutralizing antibody against CXCR3 neither changed IP-10 induced PAK-2 cleavage nor activation of JNK. In contrast, inhibition of TLR4 signaling by addition of a dominant negative virus for myeloid differentiation primary-response protein 88 (Myd88), a key adaptor molecule in TLR4 signaling, prevented both IP-10 induced PAK-2 cleavage and activation of JNK (Fig. 6-2H,I). Also, blocking Akt activation by two different PI3K inhibitors (Wortmannin and LY294002) blocked PAK-2 cleavage as well as JNK activation, which suggest that Akt might be upstream of PAK and JNK and confirms their interaction.

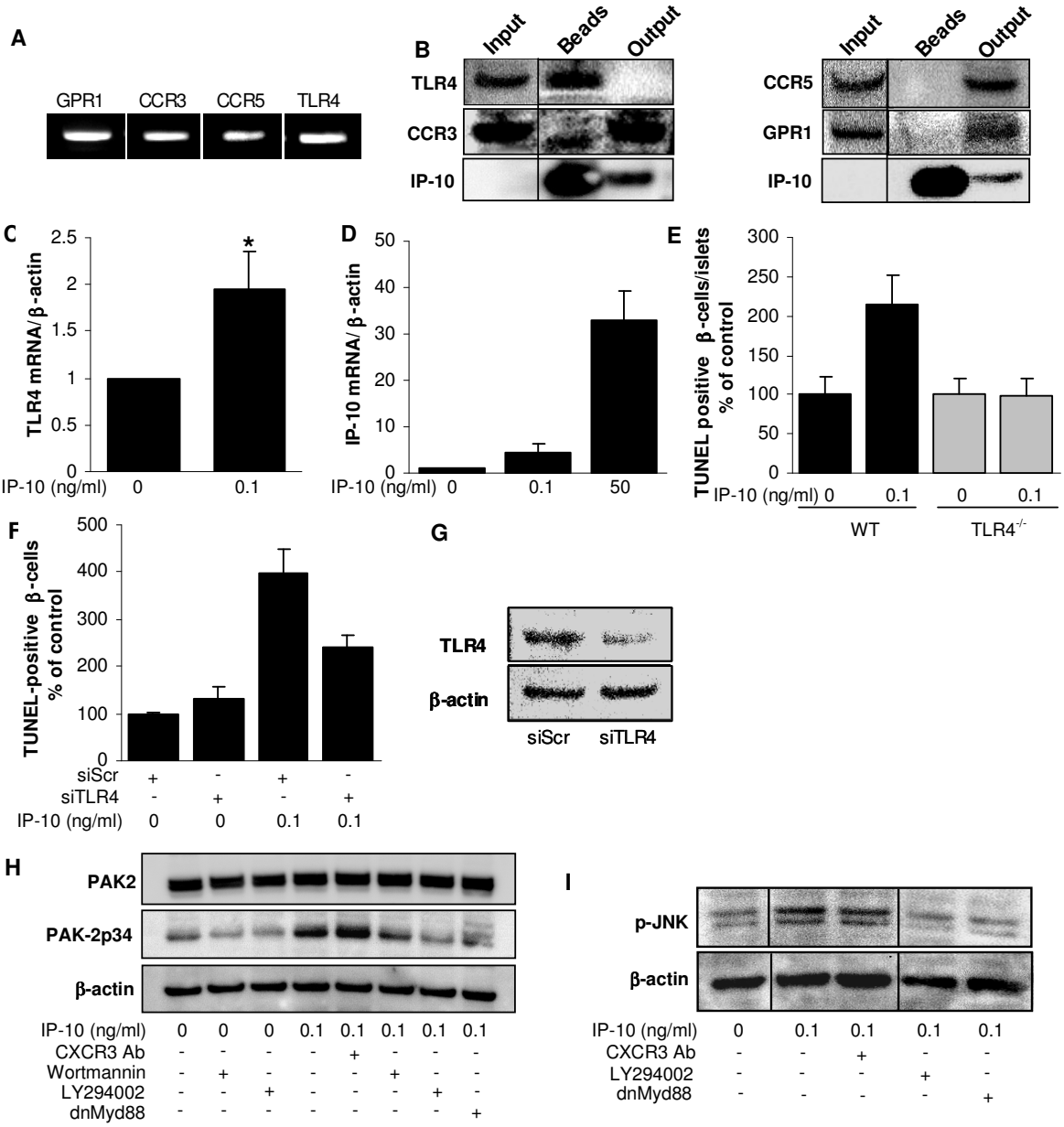

Figure 6-1. IP-10 binds to TLR4 in pancreatic β-cells and mediates apoptosis

(**A**) PCR-analysis of GPR1, CCR3, CCR5 and TLR4 in isolated human islets. (**B**) Pull down assay in human islets. IP-10 was bound to magnetic beads and incubated with human islet lysates. After magnetic separation, the bead fraction, supernatants (output) together with the original islet fraction before the pull down (input) were transferred to lysate buffers and western blot analysis was performed for TLR4, CCR3, CCR5, GPR1 and IP-10. The analysis was done in triplicate from 3 different islet donors. (**C,D**) Quantitative RT-PCR was performed in human islets exposed to IP-10 for 3 days, the increase in TLR4 (**C**) and IP-10 (**D**) mRNA expression are expressed as relative change from untreated control and represents the mean of four experiments from four islet donors. **(E)** Analysis of β-cell apoptosis in WT and TLR4$^{-/-}$ mice. Mouse islets were cultured on ECM dishes for 4 days in 5.5 mM glucose alone or in the presence of 0.1 ng/ml IP-10. **(F)** Human islets were cultured on ECM and exposed to 50 nM scrambled control siRNA (siScr) or siRNA to TLR4 (siTLR4). One day after transfection, IP-10 was added and apoptosis was measured by double-staining for TUNEL and insulin after a 4-day culture period. Results are means ± SE of the percentage of TUNEL-positive β-cells normalized to control (siScr) at 5.5 mM glucose. **(G)** Western blot analysis of TLR4 shows 48% downregulation in the siTLR4 treated islets. **(H,I)** Western blot analysis of full length PAK-2, cleaved PAK-2p34 **(H)** or p-JNK **(I)** from human islets after 24 h exposure to 0.1 ng/ml IP-10 with or without pre-incubation with an antagonistic CXCR3 antibody, 100 nM Wortmannin, 50 µM LY294002 or a dominant negative virus for Myd88 as indicated. Actin was used as a loading control. Western blots are representative of three independent experiments from three different organ donors. *p<0.05 to untreated control, **p<0.05 to IP-10 treated control islets.

Discussion

Cytokines and chemokines produced and secreted by activated macrophages, adipocytes and also by pancreatic β-cells have been suggested to initiate β-cell apoptosis in T1DM and T2DM (53). Understanding the mechanisms of secretion and function of these pro-inflammatory factors is important for treatment of diabetes.

One potential pathway involves secretion and production of pro-inflammatory signals within the islets as well as in the circulation (9; 53). Part of this pro-inflammatory cascade in the β-cell is mediated by elevated glucose concentrations. Once hyperglycemia is present, the rate of β-cell apoptosis is accelerated. When β-cells are exposed to chronic high glucose levels in vitro, or in response to viral infection (54), they produce and secrete the pro-inflammatory cytokine interleukin-1β (IL-1β) (55). Inhibition of IL-1β by its naturally occurring antagonist, the interleukin-1 receptor antagonist (IL-1Ra), blocked apoptosis and induced β-cell proliferation (55).

In the present study, we report that the chemokine IP-10 is produced and secreted by cultured human islets of patients with T2DM. Importantly, in these islets IP-10 was predominantly localized in β-cells. A diabetic milieu (high glucose, free fatty acids or h-IAPP) did not induce IP-10 synthesis in human islets in vitro indicating that IP-10 secretion is independent of glucolipotoxicity or IAPP oligomer toxicity.

In the non-obese diabetic (NOD) mouse model, IP-10 is produced in pancreatic islets before detectable insulitis (25; 56). In a model of LCMV-induced diabetes, IP-10 has been shown to be among the first chemoattractant factors expressed in the pancreas (57).

In the present study, we detected IP-10 positive β-cells in a newly diagnosed patient with T1DM. Furthermore, in a large study with humans, IP-10 serum levels were increased in patients with newly diagnosed T1DM (19) and in patients at risk of developing T2DM (21). These data suggest that up-regulated IP-10 synthesis takes place in either a pre- or in an early phase of the disease.

In confirmation with previous data (25), β-cell IP-10 expression in vitro was induced by IFN-γ alone or in a mixture with IL-1β and/ or TNF-α. Here we show that also TNF-α alone can induce IP-10 expression. TNF-α has been shown to be up-regulated under conditions of insulin resistance and T2DM (15), and thus provides a mechanism for IP-10 up-regulation in T2DM.

Macrophages producing these cytokines could be the possible paracrine inducers of IP-10 expression, since resident macrophages are present in all islets and increased in islets in T2DM (58). Another possibility is, that IP-10 up-regulates its own expression in islets as shown by RT-PCR (Fig.6D), since in T1 as well as in T2DM, IP-10 serum levels are elevated and could trigger IP-10 expression in pancreatic islets.

In our current study, we assessed consequences of such elevated IP-10 concentrations on β-cell turnover and function. Culture of human islets with IP-10 increased β-cell apoptosis as well as proliferation, with the net number of β-cells being decreased. We frequently observed doublets of apoptotic β-cells, indicative of post-mitotic apoptosis, supporting the concept that replicating β-cells are more vulnerable to apoptotic stimuli (59). While the IP-10 effect on β-cell function, proliferation and apoptosis occurred after chronic incubation, acute glucose stimulated insulin secretion was already impaired in the presence of IP-10 after 1h. A possible explanation for that early IP-10 effect on acute insulin secretion, which was only present at the stimulatory glucose concentration of 16.7 mM, could be that the combination of high glucose and IP-10 has rapid deleterious effects. IP-10 induced a ~3-fold induction of β-apoptosis in all human and mouse islet isolations, which correlates with the induction of apoptosis observed in vivo in pancreatic sections from autopsy from patients with T2DM (1) as well as with T1DM (3).

A critical regulator of β-cell proliferation and survival is Akt (also known as protein kinase B (PKB)), which is activated downstream of PI3K (60; 61). In previous reports, blocking Akt phosphorylation by the PI3K inhibitor Wortmannin or other Akt inhibitors renders islets more susceptible to β-cell apoptosis (reviewed in (62)). Moreover, transgenic mice expressing a constitutively active Akt specifically in β-cells have increased β-cell size (61), whereas mice lacking Akt have a decrease in β-cell mass (63). Insulin, acting in an autocrine manner, promotes Akt phosphorylation in β-cells in culture (64). Here we show, that IP-10 mediates its effects through Akt activation independent of insulin secretion (at physiological glucose conditions, insulin levels were unchanged in control and IP-10 treated islets). Upon chronic chemokine stimulation, this leads to impaired function and β-cell death rather than survival.
This is reminiscent of downstream signaling of pro-inflammatory cytokines. IFN-ɣ as well as TNF-α activate PI3K upon receptor binding, which phosphorylates Akt and induces increased surface expression of the Fas receptor, leading to apoptosis in vascular smooth muscle cells (65; 66). TNF-α or IFN-γ alone or in combination with IL-1β induces Fas expression in the β-cells and apoptosis (67; 68), and it is doubtless, that Fas is a major pathway of β-cell destruction in T1DM (69) and T2DM (70; 71) as well as of regulation of insulin secretion (72).

PI3K/Akt signaling leads to the transcriptional activation of IP-10 (73). Since IFN-γ induces IP-10 expression, IP-10 might induce a vicious cycle in β-cells, with IFN-γ induced Akt activation as a possible mediator of IP-10 up-regulation.
Notably, in our study sustained Akt activation by IP-10 triggered apoptosis in the β-cell. Administration of the PI3K inhibitor Wortmannin inhibited Akt phosphorylation as well as IP-10 induced β-cell death. A possible explanation for this is, that IP-10 induced Akt activation first leads to proliferation and survival, however at an event, when β-cells are highly vulnerable to apoptosis, it is finally switched towards β-cell death by Akt dependent

downstream signals. One of such downstream targets of Akt are p21-activated kinases (PAK; (74; 75)). PAKs are activated by a variety of GTPase dependent and independent mechanisms (76). Among them, PAK-1 and –2 regulate cell survival and apoptosis. PAK-1 protects from apoptosis (74; 77), whereas PAK-2 has a unique dual function: activation of full length PAK-2 stimulates cell survival (78), whereas upon cleavage of PAK-2 into a 34-kDa activated form (PAK-2p34) directs apoptosis. PAK-2 is a substrate for caspases during apoptosis in various cell types (79-81). Here we show for the first time the presence of the cleaved fragment PAK-2p34 upon caspase-3 activation in human islets as well as in isolated rat β-cells. Such proteolytic activation of PAK-2 correlated with activation of the c-Jun amino terminal kinase (JNK) in our study as well as in previous reports (75; 82). Similar to the pattern of Akt activation, we observed that stimulation of human islets with IP-10 elicits a prolonged activation of JNK.

Inflammatory cytokines and free fatty acids activate JNK in several tissues including the β-cell, leading to β-cell destruction and insulin resistance (see review (41)). The JNK pathway has been proposed as a candidate gene implicated in T2DM (83). JNK inhibition promoted β-cell survival (84) as well as insulin sensitivity *in vitro* and *in vivo* (85). Based on our results we therefore assume, that pro-apoptotic IP-10 signals involve PAK-2p34 and JNK activation.

IP-10 exerts its biological activity through the G-protein-coupled receptor CXCR3, but also CXCR3 independent effects of IP-10 have been described (43; 86; 87). We detected specific CXCR3 expression within human islets in β-cells and T-cells. However, most CXCR3 positive islet cells could not be allocated to any particular endocrine cell type, and blocking CXCR3 did not prevent IP-10 mediated apoptosis in the β-cell. Our data support the hypothesis that the IP-10 effect on pancreatic islets is CXCR3-independent. Firstly, IP-10 exhibited similar effects on the β-cells in the presence and absence/neutralization of CXCR3, and secondly, two other known CXCR3 binding chemokines I-TAC and Mig did not affect β-cell turnover. The possible mechanisms of such a CXCR3 independent IP-10 signaling would therefore be the presence of another IP-10 binding receptor. This is supported by the fact, that the low dose of IP-10 tested (0.1 ng/ml = ~ 10 pM) had full efficacy, but the dissociation constant (K_D) of IP-10-binding to CXCR3 is typically in the nanomolar range (88).

A novel functional receptor with unknown identity that binds IP-10 has been identified recently, with abundant levels in epithelial and endothelial cells (43). Unlike CXCR3, this receptor does not bind Mig or I-TAC, which would support our findings that Mig and I-TAC have no effect on the β-cells in contrast to IP-10. Possible candidates were a variety of seven-transmembrane-spanning G-protein-coupled receptors without identified ligands, but with sequence motifs shared by chemokine receptors. Of these orphan receptors we have tested GPR1, which was highly expressed in islets but failed to bind IP-10. Another candidate, APJ (89), was only marginally present in human islets (data not shown). CCR3 is

the receptor for eotaxin and several other CC chemokines and characteristic for Th2 cells. CXCR 3 ligands have shown before to bind to CCR3 but lack agonistic effects (44; 45). CCR5 is highly up-regulated by TNF-α and IFN-γ, together with up-regulation of IP-10 (46). Both, CCR3 and CCR5 were expressed in islets. While CCR3 bound to IP-10 to a limited extend, CCR5 did not.

Interestingly, we observed activation of IP-10 mRNA upon IP-10 exposure, and such IP-10 up-regulation has been observed as a downstream event upon Toll like receptor 4 (TLR4) activation (47-49). TLR4 is the receptor for LPS and is responsible for the microbial defense mechanism. Stimulation of TLR4 activates pro-inflammatory pathways and induces cytokine expression in many cell types (90). TLR4 is highly expressed in pancreatic β-cells (47; 91). After we confirmed TLR4 expression in human islets, we then tested its binding capacity to IP-10 and found IP-10 as another ligand for TLR4.

Interestingly, the dose of 0.1 ng/ml IP-10 (~10 pM) already induced intracellular signaling, whereas the dissociation constant (K_D) of LPS-binding to TLR4 is in the micromolar range (92). These data suggest that IP-10 has comparably high affinity to TLR4.

Signal transduction downstream of TLR4 is another mechanism, which induces Akt activation, This has been shown by the ligands of TLR4; LPS (93) and lauric acid (94) and results in NFκB activation and inflammation.

TLR4 depletion has been shown to prolong islet graft survival of pancreatic islets after transplantation (95). Importantly, non-endotoxine ligands of TLR4 have been identified recently. Shi et al show that free fatty acids induce TLR4 up-regulation and activation in adipocytes and macrophages to induce inflammation and trigger insulin resistance (96).

Another recent study shows that a Loss-of-function mutation in TLR4 prevents diet-induced obesity and insulin resistance (97). Furthermore, a Toll-like receptor 4 gene polymorphism in humans is associated with reduced CRP levels and a decreased risk of coronary heart disease and diabetes (98). These earlier studies and our new observations suggest that TLR4 signaling plays an important role in diabetes and insulin resistance.

The present study provides further evidence that chemokines, in addition to other pro-inflammatory factors, directly trigger β-cell destruction and contribute to the pathophysiology of T2DM. Moreover, we suggest a potential mechanism for the switch from proliferation into apoptosis in β-cells. To prevent such a progression using anti-inflammatory targets of the TLR4 signaling pathway will be of high importance to rescue the β-cell from inflammation induced self-destruction to preserve β-cell function and mass.

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7.2 ABBREVIATIONS

ADP	Adenosin diphosphate
ATP	Adenosin triphosphate
BMI	Body mass index
Ca^{2+}	Calcium
Db/db	Leptin-receptor deficient mouse model
DD	Death domain
Dia	Diazoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmatic reticulum
ERK1/2	Extracellular signal regulated kinases 1/2
FasL	Fas Ligand
Foxo	Forkhead box protein
Glut2	Glucosetransporter 2
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-1Ra	Interleukin-1 Receptor Antagonist
IL-1RAcP	IL-1R accessory protein
IL-1RI	Interleukin-1 type 1 receptor
IL-1β	Interleukin-1β
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal Kinase
JNKi	Small inhibitory peptide for JNK
K^+	Potassium
MAPK	Mitogen-activated protein kinase
mM	Millimolar
NEFAs	Non-esterified fatty acids
NGT	Normal glucose tolerance
PD	PD098059
PDX-1	Pancreatic duodenum homeobox-1
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction

Soma	Somatostatin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

7.3 ACKNOWLEDGMENTS

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Manuscript in preparation

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Poster Presentation:
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